THE ENHANCEMENT OF THE TOXICITY OF SODIUM FLUORIDE IN THE RAT BY HIGH DIETARY FAT [1]

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It has been demonstrated that the toxicity of certain fluorides which have caused fluorosis can be measurably reduced by dietary means (National Research Council, '55). Little effort, on the other hand, has been made to enhance the effect of dietary fluorides. A study has been made to determine the effect upon the intact animal (Phillips and Hart, '35) of certain metabolites involved in glycolysis in the hope that a means of circumventing the toxic action of fluorine in the animal body would be found. More recently Johnson and Lardy ('50) reported that fluorides inhibited the *in vitro* oxidation of fatty acids.

In a further effort to gain information upon the site of action of fluorine in the intact animal studies have been made to determine the effect of high-fat diets upon the development of fluorosis in the rat. These studies indicate that high-fat diets enhance the toxicity of fluorine supplied as sodium fluoride in the diet.

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EXPERIMENTAL

Female weanling rats were used for these studies. Unless otherwise stated the animals were given food and water ad libitum. They were caged individually or by groups depending upon the criteria to be applied. In all cases they were kept on raised wire screens. For the most part they were fed their respective diets for 42 to 50 days. A purified diet was

TABLE 1

Experimental diets

EXPERIMENT	INGREDIENT	LOT NO.					
		1	2	3	4	5	6
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
I (5 rats/lot)	Sucrose	72	67	57	72	67	57
	Casein	19	19	19	19	19	19
	Cottonseed oil	5	10	20	5	10	20
	Salts IV	4	4	4	4	4	4
	NaF	0.0	0.0	0.0	0.1	0.1	0.1
II (5 rats/ lot)	Sucrose	75	52.5	71	48.5	67	44.5
	Casein	16	16	20	20	24	24
	Celluflour	0	12.5	0	12.5	0	12.5
	Cottonseed oil	5	15	5	15	5	15
	Salts IV	4	4	4	4	4	4
	NaF	0.1	0.1	0.1	0.1	0.1	0.1
III (10 rats/lot)	Sucrose	67	44.5	67	44.5	(Lots 5–10	
	Casein	24	24	24	24	used ration	
	Celluflour	0	12.5	0	12.5	4 supple-	
	Cottonseed oil	5	15	5	15	mented as	
	Salts IV	4	4	4	4	indicated	
	NaF	0.0	0.0	0.1	0.1	below.)[1]	
IV	Sucrose			67	44.5		
	Casein			24	24		
	Celluflour			0	12.5		
	Cottonseed oil			5	15		
	Salts IV			4	4		
	NaF			0.15	0.15		

[1] Supplements to the diets fed the various groups in experiment III were as follows: lot 5, 200 mg of Ca-pantothenate/100 gm of ration; lot 6, 1% of DL-methionine; lot 7, 5 mg of ascorbic acid/kg body wt./day; lot 8, 200 mg of Ca-pantothenate and 1% of DL-methionine; lot 9, Ca-pantothenate, ascorbic acid and methionine as used in the preceding lots; lot 10, cottonseed oil replaced by butter oil.

used as the basal ration in all cases, and it was modified to meet the conditions of the experiments involved (table 1).

In experiment IV the 5 rats in lot 3 were injected daily, subcutaneously, with 0.3 ml of physiological saline solution. The 16 rats in lot 4 were given their respective treatments (two rats per treatment) as follows: 0.3 ml of physiological saline, subcutaneously; 10 mg of the di-sodium salt of adenosine triphosphate (ATP·Na_2), subcutaneously; 10 mg of ATP·Na_2, intraperitoneally; 10 mg of Ca-pantothenate, subcutaneously; 10 mg L-cysteine·HCl, subcutaneously; 10 mg of ATP·Na_2 and 10 mg of cysteine, subcutaneously; 10 mg of ATP·Na_2 and 10 mg of Ca-pantothenate, subcutaneously; injection of all three compounds, subcutaneously. The ATP·Na_2 cysteine and Ca-pantothenate were each made up in distilled water so that 0.1 ml of solution contained 10 mg of the compound.

The composition of the vitamin mix fed to each lot in all the experiments was as follows in milligrams per kilogram of ration: choline chloride 1000; inositol 100; Ca-pantothenate 20; niacin 10; riboflavin 5; thiamine hydrochloride 5; pyridoxine hydrochloride 3; folic acid 0.2; biotin 0.1; vitamin B_{12} 0.01. Vitamins A, D, E and K were provided in the form of haliver oil fortified with α-tocopherol and menedione (2 drops each week).

Salts IV (Phillips and Hart, '35) was used as the source of minerals. At the close of each experiment, except no. IV, a sufficient number of femurs were removed from rats of each lot to allow for fluorine determination according to the method of Willard and Winter ('33) as modified by the Alcoa Research Laboratories ('47). The bones were dehydrated, fat extracted and then ashed at 550°C. for two and one-half hours. In experiment I the rats were fed an amount of food equal to the average food consumption of the lot eating the least. Thereafter the diets were made isocaloric by the inclusion of celluflour in the high-fat mixtures.

RESULTS AND DISCUSSION

The results obtained in experiment I are summarized in table 2. An increase in the fat content of the basal ration above 5% caused, in the absence of added fluorine, a slight stimulation in the rate of growth of the rat. If, however, the dietary fat was increased in the presence of 0.1% of added dietary sodium fluoride the growth rate of the rats was markedly retarded. The retardation effect was obtained with 10 or 20% added fat.

It is well known that the inclusion of 0.1% of sodium fluoride in the diet will inhibit growth. This is due to a reduced food

TABLE 2

The accentuating effect of high dietary fat levels on fluorosis

FAT	NaF	GAIN [1]		MG OF FLUORINE PER FEMUR [2]	FLUORINE ASHED WT. BASIS [2]
		Average	Range		
%	%	*gm*	*gm*	*mg*	*ppm.*
5	0	108	(95–114)	...	...
10	0	113	(102–118)	.023	172
20	0	121	(120–127)	...	...
5	0.1	99.5	(93–104)	1.58 [3]	12,500 [4]
10	0.1	74.5	(71–116)	1.82 [3]	17,500 [4]
20	0.1	74.5	(62–83)	1.90	16,350

[1] Average of 5 rats per group.
[2] Average of three animals per group.
[3] The difference is significant at the 5% level.
[4] The difference is highly significant (1% level).

intake. In the experiments summarized in table 2 all of the rats in their respective lots received the same amount of food daily and hence equal amounts of fluorine. The data in table 2 indicate that the absolute amount of fluorine per femur was increased by the increased dietary fat as well as the fluorine concentration on an ashed weight basis. Because of the protein-energy factors involved in experiment I a second experiment was carried out, wherein isocaloric diets were used. The results are summarized in table 3. The data show that increasing the casein content of the diet from 16 to 24% had no favorable effect on the development of fluorosis. As a

matter of fact the 24% protein level seemed to enhance fluorine toxicity to a greater extent then did the 16% level of protein. It was again evident that the inclusion of 15% fat in the basal ration increased the toxicity of sodium fluoride fed at the 0.1% level.

Coenzyme A determinations on the liver of these experimental rats failed to show any significant difference due to treatments. Tests for acetone bodies in the urine of the experimental animals were negative.

An effort was then made to determine if certain dietary co-factors involved in fatty acid oxidation were involved in

TABLE 3

The influence of protein level on the high dietary fat accentuation of fluorosis (5 animals per group)

FAT	CASEIN	NaF	GAIN	
			Average	Range
%	%	%	*gm*	*gm*
5	16	0.1	118	(106–142)
15	16	0.1	93.5	(89–98)
5	20	0.1	120	(109–132)
15	20	0.1	77	(49–92)
5	24	0.1	135	(124–137)
15	24	0.1	51	(33–67)

the enhancement effect of the high fat diet. The results of this experiment are summarized in table 4. Again it is apparent that the feeding of 15% of fat in the diet which contained 0.1% of sodium fluoride depressed the growth rate. Supplementation with calcium-pantothenate, methionine or ascorbic acid, either alone or in combination, failed to ameliorate the fluorosis caused by added dietary fat. When butter oil replaced cottonseed oil, no difference in the growth rate could be detected.

The average fatty acid chain [illegible]th of butter fat is less than that of cottonseed oil, and th[illegible]ore would require fewer steps in its catabolism. If fluorine inhibited only the steps involved in the breakdown of the long-chain fatty acids to

TABLE 4

The effect of various dietary supplements upon the accentuation of high dietary fat upon the development of fluorosis (10 animals per group)

FAT	SUPPLEMENTS	NaF	GAIN		FLUORINE PER FEMUR	FLUORINE ASHED WT. BASIS
			Average	Range		
%		%	*gm*	*gm*	*mg*	*ppm.*
5	None	0	126	(113–150)	.015	92
15	None	0	121.8	(105–133)	.016	125
5	None	0.1	98.6	(85–112)	1.52	14,480 [1]
15	None	0.1	54.5	(33–71)	1.20	16,230 [1]
15	Calcium pantothenate, 200 mg %	0.1	49.1	(35–67)	1.17	17,100
15	DL-Methionine, 1% of ration	0.1	58.0	(41–72)	1.21	16,430
15	Ascorbic acid, 5 mg/kg body wt.	0.1	54.8	(35–78)	1.32	17,000
15	Calcium pantothenate and DL-methionine	0.1	56.0	(48–77)	1.14	15,400
15	Ascorbic acid, DL-methionine, calcium pantothenate	0.1	56.8	(33–77)	1.26	14,590
15	Butter oil replaced cottonseed oil	0.1	50.2	(20–64)	1.11	15,100

[1] The difference is significant at the 5% level.

those of shorter chain lengths, an animal fed fluorine should tolerate higher levels of butter fat than one fed cottonseed oil. Fifteen per cent of butter oil was equally as toxic as 15% of cottonseed oil; thus the type of fat caused no difference in the growth-retarding effect of high-fat fluorine-containing diets.

A 4th experiment was conducted to determine if injections of the various co-factors might reduce the effect of dietary fat on sodium fluoride included in the diet at the 0.15% level. Two control lots of rats were used, one of which was fed a 5% fat diet and the other 15%. Each lot of control animals was given subcutaneous injections of physiological saline daily. The animals (two per lot) in the remaining lots were treated as indicated in the experimental outline above. Neither subcutaneous nor intraperitoneal injections of the co-factors, ATP, pantothenic acid, cysteine, ATP and cysteine or ATP and pantothenic acid or a combination of all three compounds alleviated the sodium fluoride toxicity in the presence of added dietary fat.

SUMMARY AND CONCLUSIONS

1. Increasing the dietary fat from 5 to 15%, in a purified ration increased the growth-retarding effect of a diet which contained 0.1% of sodium fluoride. It was established further that the protein-energy relationship was not responsible for the retardation effect of high fat on rats fed sodium fluoride.

2. Dietary supplements of pantothenate, methionine or ascorbic acid, alone or in combination, did not alter the growth-retarding effect of added dietary fat when the ration contained 0.1% of sodium fluoride. Neither did the subcutaneous or intraperitoneal injection of ATP, pantothenate or cysteine, either singly or in combination, prevent the enhancing effects of added fat on the development of fluorosis in the rat.

3. Fifteen per cent of butter fat as well as 15% of cottonseed oil were equally effective in enhancing the toxic action of 0.1% of sodium fluoride in the ration of the rat. The en-

hancing effect of added dietary fat could not be attributed to the chain length of the component fatty acids of the fat fed.

4. In these experiments the feeding of increased levels of fat and diets containing 0.1% sodium fluoride caused an increased deposition of fluorine in the femurs.

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THE BIOLOGICAL VALUE OF OILS AND FATS

I. GROWTH AND FOOD INTAKE ON FEEDING WITH NATURAL OILS AND FATS

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It has been shown by different investigators that the growth of young rats fed butter-containing diets is better than if the butter is replaced by certain vegetable oils or fats (Schantz et al. ['40], using maize, soybean, cottonseed and coconut; Harris and Rosenfeld ['40], who used coconut and olive oil; Boer ['41], who employed olive and rapeseed oils; Freeman and Ivy ['42], coconut; Parrish et al. ['46], maize; Deuel et al. ['48], rapeseed oil; Euler et al. ['48], rapeseed oil; Nieman et al. ['52], groundnut oil).

In contradiction to these results a number of workers report that no superior growth could be observed in rats fed butter-containing diets (Deuel et al. ['44; '49], maize, cottonseed, olive, groundnut and soybean oil; Henry et al. ['45], groundnut, maize, cottonseed and soybean oil; Zialcita and Mitchell ['45], maize oil; Lassen and Bacon ['49], olive and cottonseed oil).

In the present investigation the growth-action of a large number of fats and oils has been studied. Since previous experiments had shown that the growth depends on the fat-content in the diet (Hoagland and Snider ['40; '41], Boutwell et al. ['43], Forbes et al. ['46], Barki et al. ['50]), each fat was investigated at different dosage levels, varying from 10 to 73 Cal. %.

EXPERIMENTAL

For each fat a series of 8 groups, each consisting of 8 to 12 male 21-day-old Wistar-strain rats, was used. Occasionally the number of groups was smaller than 8. All groups belonging to the same test fat series were started simultaneously, but each test fat was investigated at a different point of time.

Seven of the 8 groups received a diet in which the test fat was mixed in amounts of 10, 20, 30, 40, 50, 60 and 73 Cal. %, respectively. The 8th group served as a reference standard; its diet contained 20 Cal. % of summer butterfat. The composition of the diets used for the investigation of a given test fat is recorded in table 1.

TABLE 1

Composition of the diets

DIET	RICE	CASEIN	YEAST	SALTS	BUTTER FAT	TEST FAT	CALORIES PER GRAM
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	
C20-Summer butterfat	157.6	34.5	28.5	8.5	21.5	...	3.99
C10-test fat	187.8	32.1	28.5	8.5	...	10.8	3.74
C20-test fat	157.6	34.5	28.5	8.5	...	21.5	3.99
C30-test fat	127.4	36.9	28.5	8.5	...	32.3	4.28
C40-test fat	97.2	39.4	28.5	8.5	...	43.0	4.62
C50-test fat	67.0	41.8	28.5	8.5	...	53.8	5.01
C60-test fat	36.8	44.2	28.5	8.5	...	64.5	5.48
C73-test fat	...	47.2	28.5	8.5	...	77.6	6.18

All of the diets contained the same amounts of protein, water-soluble vitamins (dried brewers' yeast), and salts per calorie unit; e.g. 230 Cal. protein, 28.5 gm yeast, and 8.5 gm salt-mixture per 1000 Cal. The content of test fat in the diets was varied at the expense of the carbohydrate content on a caloric base. Food and drinking water were given ad libitum; the diets were prepared twice a week. The fat-soluble vitamins were administered in the form of a prophylactic dose. At the beginning of the experiment and again after 4 weeks the animals were fed by pipette 0.05 ml of a concen-

trate containing 2000 I.U. vitamin A, 12.5 μg vitamin D_2, and 10 mg tocopherol-acetate, dissolved in groundnut oil. The animals were weighed weekly in the course of the 6-week experiment. The quantity of food supplied was also determined, but the wastage was not taken into account.

RESULTS

Twenty different oils and fats have been studied. Two of them, e.g. butter and whale oil, were investigated in duplicate. The results of the increase in weight after one, three and 6 weeks are shown in table 2.

The data obtained with the two summer-butterfat groups show that the rate of growth remains practically unchanged when the fat content of the diets is increased. Only in the first few weeks and at the highest dosage, viz. 73 Cal. % summer butterfat, is the growth somewhat less than at the lower dosages; but at the end of the experiment this difference has disappeared. With the other fats, however, with the exception of lard, there was, to a greater or lesser extent, a decrease of the rate of growth with increasing fat dosages.

In order to compare the growth-promoting effect of various fats with each other and in particular with summer butterfat, the difference between a test group and the simultaneously started standard group (20 Cal. % summer butterfat) was considered a suitable criterion. The comparison with butterfat was facilitated by deducting the mean value of the two butterfat series from the differences obtained. The growth-promoting value of summer butterfat was thus zero.

In table 3 a survey is given of the results obtained after statistical treatment of the experimental data. Only the observations after three weeks are recorded. The order in which the various fats are placed is determined by the magnitude of the differences as compared with summer butterfat. Since the growth of the rats in the butterfat groups after three weeks is about 100 gm, the numerical values in the table represent approximate percentages.

TABLE 2

Mean increase in weight after 1, 3 and 6 weeks (gm)

Cal. %	Lard				Summer butterfat I				Summer butterfat II			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	24	99	195	12	27	107	206	12	25	100	196
20	10	22	100	199	..	..	...	...	..	..	...	...
30	12	25	109	216	12	26	109	218	12	25	104	211
40	12	25	107	211	12	27	108	211	12	25	105	206
50	12	25	105	218	12	25	111	220	12	24	105	218
60	12	24	104	220	12	26	110	211	12	24	103	217
73	12	23	105	220	12	21	103	216	12	21	99	208
Standard [1]	12	22	97	194	12	28	111	212	12	25	104	205

Cal. %	Olive oil				Cottonseed oil				Winter butterfat			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	..	..	...	...	12	23	101	202	12	20	79	153
20	12	24	103	201	11	21	98	202	12	19	81	171
30	..	..	...	...	12	24	103	209	12	19	82	171
40	12	22	96	195	12	24	107	217	12	15	80	176
50	..	.	.	.	12	22	97	204	12	18	78	170
60	12	19	99	203	11	21	99	213	12	14	78	177
73	12	17	90	185	12	17	87	196	12	13	70	181
Standard [1]	12	24	102	195	12	24	101	209	12	18	80	166

Cal. %	Beef fat				Shea butter				Maize oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	28	108	208	12	25	95	179	9	21	93	188
20	10	26	105	196	12	24	95	178	9	23	98	199
30	12	28	108	210	12	22	89	169	9	22	94	192
40	12	26	105	202	12	21	90	171	9	23	99	202
50	12	27	106	210	12	20	87	169	9	25	104	216
60	12	27	106	209	12	20	89	169	9	21	92	195
73	12	18	93	201	12	19	88	174	8	16	74	181
Standard [1]	9	28	111	213	12	22	93	181	10	22	94	189

Cal. %	Soyabean oil				Groundnut oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	11	26	102	190	10	25	107	214
20	11	26	100	184	10	24	104	215
30	11	26	103	198	10	25	109	219
40	11	24	92	184	9	22	102	210
50	11	24	95	188	10	22	105	215
60	11	23	97	200	10	21	99	210
73	11	17	82	184	10	14	84	193
Standard [1]	10	25	102	192	10	24	106	219

[1] Twenty Cal. % summer butterfat.

TABLE 2 *(continued)*

Mean increase in weight after 1, 3 and 6 weeks (gm)

Cal. %	Palm fat				Sunflowerseed oil				Poppyseed oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	9	27	106	204	11	26	102	204	11	24	98	197
20	9	28	104	203	12	26	103	208	11	24	98	199
30	9	30	110	207	11	25	101	208	12	24	101	207
40	9	29	110	212	12	24	103	207	11	24	99	202
50	8	27	107	214	12	24	101	214	11	24	101	207
60	8	23	100	197	10	21	88	191	11	20	89	200
73	9	16	91	193	12	17	75	177	11	13	69	177
Standard [1]	9	29	111	218	12	26	105	204	11	25	104	208

Cal. %	Coconut fat				Sesame oil				Owalanut oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	25	98	196	11	22	89	179	12	23	96	...
20	12	23	95	195	10	24	97	192	12	21	89	...
30	12	24	98	197	11	21	90	186	12	22	92	...
40	12	22	92	191	10	20	86	183	12	18	85	...
50	12	21	92	198	11	19	81	182	12	18	81	...
60	11	18	83	182	11	19	76	173	12	15	75	...
73	11	13	65	156	9	10	49	139	12	6	31	...
Standard [1]	12	26	102	203	11	22	91	190	12	22	94	...

Cal. %	Whale oil I				Whale oil II				Herring oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	11	25	99	187	10	24	97	201	12	29	107	209
20	12	23	85	169	10	20	84	181	12	23	96	189
30	11	22	85	168	10	20	85	178	12	22	90	180
40	11	20	82	163	10	20	80	177	12	19	82	164
50	11	19	74	141	10	18	76	175	12	14	70	151
60	12	14	61	149	11	13	67	168	12	12	60	127
73	11	6	47	135	8	6	48	158	12	5	44	104
Standard [1]	11	23	97	186	10	24	100	214	12	29	108	212

Cal. %	Rapeseed oil				Kapokseed oil				
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	
10	8	21	93	183	12	16	88	187	
20	10	22	81	154	12	15	73	169	
30	9	19	75	149	11	12	57	142	
40	9	13	56	131	12	7 animals died (mean 18 days)			
50	9	12	52	125	12	9 animals died (mean 15 days)			
60	9	7	35	96	12	all animals died (mean 14 days)			
73	9 (all animals died, mean 17 days)				11	all animals died (mean 13 days)			
Standard [1]	9	24	94	177	11	24	104	212	

[1] Twenty Cal. % summer butterfat.

Table 3 shows that the difference between growth with summer butterfat and the other oils and fats increases with increasing fat content in the diet. Since the growth-promoting effect of summer butterfat is practically independent of the dosage, the augmentation of the difference is actually due to a lowering of the rate of growth caused by the different oils and fats when their dosage is increased.

TABLE 3

Difference in growth-promoting value (gm) as compared with summer butterfat (3rd week)

GROUP	TYPE OF FAT	CALORIC PERCENTAGE TEST FAT IN THE DIET						
		10	20	30	40	50	60	73
1	Lard	+5	+3	+12	+10	+7	+7	+14
2	Summer butterfat (Av.)	0	0	0	0	0	0	0
3	Olive oil	–	+1	–	−6	–	−4	−6
	Cottonseed oil	+4	−3	+3	+7	−5	−1	−8
	Winter butterfat	0	−1	0	−2	−5	−4	−6
	Beef fat	+3	−3	+1	−3	−3	−2	−10
	Shea butter	+3	−1	−6	−5	−9	−6	−2
4	Maize oil	+2	+4	0	+5	+8	−2	−15
	Soybean oil	+5	0	+3	−9	−6	−3	−12
	Groundnut oil	+3	−3	+2	−6	−4	−8	−18
	Palm fat	−1	−6	0	0	−4	−9	−13
5	Sunflowerseed oil	0	−2	−4	−2	−5	−17	−24
	Poppyseed oil	−2	−5	−2	−4	−4	−14	−29
	Coconut fat	0	−5	−2	−9	−9	−17	−30
6	Sesame oil	−2	+2	−4	−8	−15	−18	−50
	Owalanut oil	+5	−5	−2	−9	−14	−19	−58
7	Whale oil (Av.)	+3	−14	−13	−17	−24	−35	−45
	Herring oil	+1	−12	−19	−27	−40	−48	−59
	Rapeseed oil	−3	−18	−24	−44	−49	−64	†
8	Kapokseed oil	−13	−31	−46	†	†	†	†

From a statistical analysis[1] of the figures it was found that, except for random sampling variations, the variability of the increase in weight after three weeks was the same for all groups. This quantity appears to be independent of the growth rate, the season, and the number in the litter. The best estimate for the variance of the increase in weight in the third week, derived from 1983 observations was found to be $\sigma^2 = 118.1911$. Moreover the analysis showed that the distribution may be considered normal. The values in table 3 which differ significantly ($P = 0.05$) from those of the corresponding summer-butterfat group are boxed in.

In table 3 the various oils and fats are grouped according to the results of the significance test.

This classification of the different oils and fats is fairly artificial and is of value only for purpose of orientation. The fats of group 3, for example, do not differ significantly from summer butterfat, but they were grouped separately, since the differences with respect to summer butterfat at dosages of 50, 60, and 73 Cal. % all have a negative sign. Moreover, their total mean at a dosage of 73 Cal. % differs significantly from summer butterfat.

The further analysis of the gain data concerns the type of the growth curve. It has been found that the increase in weight when the standard diet with 20 Cal. % summer butterfat is administered is practically linear for the first 6 weeks. The growth index is $\frac{\text{increase in weight 3–6 weeks}}{\text{increase in weight 0–3 weeks}} = 0.985$ for a series of 37 standard groups.

The standard error of this mean $= 0.092$, so that the fiducial limits for $P = 0.05$ are: 0.789 and 1.151.

With the aid of these values it is possible to establish which growth indices deviate significantly from the normal standard (20 Cal. % summer butterfat). Table 4 records these growth indices. The values which deviate significantly are italicized.

[1] The statistical problems were studied in collaboration with the "Mathematisch Centrum," Amsterdam. In this connection I wish to thank especially Professor Dr. J. Hemelrijk and his co-workers.

TABLE 4

Growth indices and efficiency constants

TYPE OF FAT	GROWTH INDICES $\left(\frac{\text{INCREASE IN WEIGHT 3–6 WEEKS}}{\text{INCREASE IN WEIGHT 0–3 WEEKS}}\right)$								EFFICIENCY CONSTANTS $\left(\frac{\text{INCREASE IN WEIGHT IN 6 WEEKS}}{\text{FOOD CALORIES IN 6 WEEKS}} \times 100\right)$								
	Standard (20 Cal. %) summer butterfat	Caloric percentage test fat in the diet							Standard (20 Cal. %) summer butterfat	Caloric percentage test fat in the diet							
		10	20	30	40	50	60	73		10	20	30	40	50	60	73	mean
Lard	1.09	0.97	0.99	0.98	0.97	1.08	1.12	1.10	7.13	7.84	7.65	7.56	6.71	7.46	7.07	6.90	7.29
Summer butterfat I	0.91	0.93	0.91	1.00	0.95	0.98	0.92	1.10	8.21	*9.80*	8.21	8.23	8.25	8.55	7.60	8.10	8.37
II	1.01	0.96	1.01	1.03	0.96	1.08	1.11	1.10	8.24	7.52	8.24	7.73	8.25	7.64	7.89	8.00	7.94
Olive oil	0.91	...	0.95	...	1.03	...	1.05	1.06	...	...	...	...	...	...	...	...	...
Cottonseed oil	1.05	1.00	1.06	1.03	1.03	1.10	1.15	*1.25*	8.14	8.00	7.68	8.94	8.53	8.80	8.29	8.32	8.34
Winter butterfat	1.08	0.94	1.11	1.09	*1.20*	*1.18*	*1.27*	*1.59*	6.29	6.69	6.33	6.90	6.40	7.15	6.91	8.15	6.85
Beef fat	0.92	0.93	0.87	0.94	0.92	0.98	0.97	*1.16*	7.54	8.36	7.13	*9.24*	7.71	7.24	6.89	7.40	7.69
Shea butter	0.95	0.88	0.87	0.90	0.90	0.94	0.90	0.98	6.40	7.10	*6.20*	6.32	*5.41*	*5.94*	*6.01*	7.15	6.32
Maize oil	1.01	1.02	1.03	1.04	1.04	1.08	1.12	*1.45*	7.94	8.70	8.52	8.54	8.93	9.09	8.83	8.52	8.64
Soybean oil	0.88	0.86	0.84	0.92	1.00	0.98	1.06	*1.24*	7.58	8.02	6.97	8.52	7.37	8.01	8.32	8.25	7.88
Groundnut oil	1.07	1.00	1.07	1.01	1.06	1.05	1.12	*1.30*	8.23	8.82	8.50	9.05	8.43	8.26	8.76	8.51	8.57
Palm fat	0.96	0.92	0.95	0.88	0.93	1.00	0.97	1.12	9.20	9.21	*9.25*	*9.42*	8.43	*9.33*	7.85	8.63	8.92
Sunflowerseed oil	0.94	1.00	1.02	1.06	1.01	1.12	*1.17*	*1.36*	8.13	7.39	8.43	8.74	*9.33*	7.50	7.95	8.17	8.21
Poppyseed oil	1.00	1.01	1.03	1.05	1.04	1.05	*1.25*	*1.57*	8.24	8.36	8.14	9.17	7.81	8.44	9.10	8.53	8.47
Coconut fat	0.99	1.00	1.05	1.01	1.08	1.15	*1.19*	*1.40*	8.31	8.20	8.04	8.83	7.50	8.14	7.81	8.07	8.11
Sesame oil	1.08	1.00	0.99	1.08	1.13	*1.24*	*1.27*	*1.84*	6.82	6.85	7.02	7.24	7.40	7.59	7.28	7.48	7.21
Owalanut oil	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Whale oil I	0.91	0.89	0.99	0.98	0.99	0.91	*1.44*	*1.87*	7.38	...	...	7.36	7.84	7.09	7.86	8.33	7.64
II	1.14	1.07	1.15	1.09	*1.21*	*1.30*	*1.51*	*2.29*	8.34	8.33	7.67	7.88	7.87	7.83	8.22	...	8.02
Herring oil	0.96	0.95	0.97	1.00	1.00	*1.16*	1.12	*1.56*	7.55	8.22	6.99	7.68	6.55	7.20	6.58	6.72	7.19
Rapeseed oil	0.88	0.97	0.90	0.99	*1.34*	*1.40*	*1.74*		7.30	7.48	6.45	6.82	7.37	7.72	6.91	...	7.15
Kapokseed oil	1.04	1.13	1.32	1.49					7.67	8.03	7.42	7.26	...	...	...	...	7.60
Mean	0.985 ± 0.092								7.73 ± 0.71	8.05	7.62	8.07	7.69	7.84	7.69	7.95	7.83

Table 4 shows that a number of growth indices differ significantly from the standard, all these deviating values being too large. The data indicate that the deviation from the standard is greater as the rate of growth is smaller. The correlation between the increase in weight in the first three weeks and the magnitude of the growth indices appears to be highly significant ($r = -0.70$). Thus the type of the growth curve is generally linear (growth index $= \pm 1$), but in the case of certain oils and fats with little growth-promoting action, given in a large dose, the rate of growth in the second half of the experiment is better than in the first half. A certain "adaptation" manifests itself, which becomes more pronounced as the growth rate of the animals is diminished.

The data concerning the food intake refer to the total amount of food supplied in 6 weeks to each group. As already mentioned, the wastage of food, which will certainly have occurred and to a varying extent for the different groups, is not taken into account. As was to be expected, there is a positive correlation between the food intake and the increase in weight. For this reason the efficiency constants were calculated. Efficiency constant $= \frac{\text{increase in weight in 6 weeks}}{\text{food calories in 6 weeks}} \times 100$. The second part of table 4 records these efficiency constants. The average of the efficiency constants of the standard groups (20 Cal. % summer butterfat) is 7.73 with S.D. $= 0.71$. Values which differ significantly from this standard mean are in italics. With the exception of shea butter (too low) and palm oil (too high) the table suggests that the efficiency constants are approximately the same for all the fats and oils. It seems therefore that the increase in weight per calorie of food intake is independent of the type of fat and the level at which it is administered.

Finally, some data are given on the mortality observed in some of the experimental groups. Of the group which received the diet with 73 Cal. % of rapeseed oil all animals died, after 8, 10, 12, 14, 15, 20, 20 and 37 days, respectively (mean 17 days).

The mortality in the kapokseed oil groups was as follows:
40 Cal. %: 7 of the 11 animals died after 6, 14, 18, 18, 21, 21 and 27 days, respectively (mean 18 days).
50 Cal. %: 9 of the 12 animals died after 11, 11, 12, 15, 16, 16, 17, 19 and 20 days, respectively (mean 15 days).
60 Cal. %: all animals died after 8, 10, 13, 14, 15, 15, 16, 16, 17, 18, 19 and 21 days, respectively (mean 14 days).
73 Cal. %: all animals died after 8, 9, 11, 12, 12, 12, 13, 14, 15, 15 and 20 days, respectively (mean 13 days).

DISCUSSION

The 20 oils and fats investigated do not all have the same effect on growth, but show certain differences. It is illogical to explain this by the theory of the presence of an unknown "growth-promoting" substance in butter. For in that case the favourable substance should not only occur in butter but also in the other oils and fats, the content diminishing with decreasing growth-promoting effect. As a consequence, on raising the dosage with these oils and fats an improvement of the growth should be expected, while, on the contrary, a decline has been observed. Moreover, the "deficiency," which will develop by depletion of the growth-promoting substance, starts at the beginning of the experiment so that a so-called "run-out time" is missing. Besides, the values of the growth indices of the oils and fats with low growth-promoting effect are higher than 1, which points to a certain improvement where an aggravation could be expected. All these facts argue against the growth-promoting hypothesis.

In contrast to this, a growth-retardation theory in which the presence of "growth-retarding" substances in various oils and fats is assumed, explains both the lowering of the rate of growth on raising the fat content in the diet as well as the growth differences obtained with the various oils and fats investigated. The poorest-acting oils and fats should contain the largest amounts of retarding substance(s) and the "retarding potency" should decrease as the growth-action of the fat improves. This theory has received support in the

finding that erucic acid, which is present to the extent of about 50% in rapeseed oil, was responsible for the poor growth-action of this oil (Thomasson and Boldingh, '55).

Nothing is known about the nature of any growth-retarding substance in the other poorly-acting oils and fats. Assuming them to be fatty acids, attention is focused on the long-chain fatty acids with 20 or more carbon atoms. Leaving the degree of unsaturation out of account, owalanut oil contains 23% of 20-carbon atom acid, whale oil and herring oil 20 and 52% respectively of 20- and 22-carbon atom acids, and rapeseed oil 50% of 22-carbon atom acid. On this basis the hypothesis is suggested that fatty acids with 20 or more carbon atoms have an unfavourable influence on the growth of young animals. Unfortunately, the composition of kapokseed oil is not well-known; otherwise further suggestions could possibly be obtained from this quarter.

The results indicate that the various oils and fats, with the exception of shea butter and palm oil, seem to have the same food efficiency in spite of differing values in promoting growth. Therefore, it is probable that irrespective of the kind of fat and the level at which it is administered, the caloric value for the animal is the same; and moreover that the growth-retarding fatty acid will be metabolized to the same extent and in the same way as the non-retarding ones, but that only the *rate* at which metabolism takes place is different for the various fatty acids. The low caloric value of shea butter may be a result of the high content of unsaponifiable matter (5 to 7%). The cause of the high value of the efficiency constant of palm oil is not known.

The fact that the food intake of the rat can be regulated by means of the type of fat opens up the possibility of a practical application in medicine, insofar as this rule also holds good for man. In particular, a fat which lowers the food intake will have a chance in the treatment of those forms of obesity which are due to excessive food intake. This problem is under further investigation.

The growth and food intake of the animal form a generally accepted criterion for judging its state of health and a retarded rate of growth is considered as an expression of a pathological condition, or a dysfunction. On this basis the biological value of the oils and fats with a good growth-promoting effect will be rated higher than those with a poor action. This view seems to be supported by the fact that the animals on diets containing high dosages of rapeseed oil or kapokseed oil died within the 6-week experimental period. This conception, however, is incorrect, as has become apparent from a further investigation (Thomasson, '55). It appeared that rats fed 50 Cal. % of rapeseed oil lived significantly longer than rats on a diet containing 50% summer butterfat, although the growth rate, and the daily food intake, of the rapeseed oil animals were much poorer than those of the animals fed butterfat. This observation indicates that growth-inhibiting substances in oils and fats need not of necessity have an adverse effect, but can have a favourable influence instead. In addition, the longer life span observed supports the conception that growth-retarding substances can be useful in the therapy of obesity. Finally, this investigation argues strongly against the existence of an unknown vitamin in butter. For, now that it has appeared that animals receiving this factor — the butter group — have a shorter life span than the "deficient" rapeseed oil animals, the existence of such an unknown butter factor becomes highly dubious.

SUMMARY

In 6-week feeding experiments with rats the behaviour of 20 oils and fats has been investigated, each of them being administered in 7 different dosages from 10 to a maximum of 73 Cal. %.

The various oils and fats showed differences in growth-promoting effect. Nevertheless, the food-efficiency seemed to have a constant value and to be independent of the type of fat used. Only shea butter and palm oil were exceptions to this rule.

As an explanation of the differences observed the presence of growth-inhibiting substances is suggested. Such substances need not be considered as harmful, but may possibly even exert a favourable influence, judging from the data on longevity.

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THE BIOLOGICAL VALUE OF OILS AND FATS

II. THE GROWTH-RETARDING SUBSTANCE IN RAPESEED OIL

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TWO FIGURES

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INTRODUCTION

Previous work (Thomasson, '55) has shown that the growth of young rats depends on the type of fat in the diet. On the basis of theoretical considerations it was supposed that the growth differences are caused by the presence of growth-inhibiting substances in oils and fats with a poor growth-promoting effect. In particular, attention was focussed on the fatty acids with 20 or more carbon atoms, since these acids occur in a number of oils and fats associated with retarded growth. Rapeseed oil, for example, which shows a pronounced growth-retarding action, contains about 50% of erucic acid ($\Delta^{13,14}$-docosaenoic acid), and hence the possibility was considered that the latter acid would be responsible for the poor growth response obtained with rapeseed oil.

Von Beznàk et al. ('43a) were of the opinion that the growth-retarding action of rapeseed oil is not caused by erucic acid, but by some toxic compound. In the course of their investigations Von Beznàk et al. ('43b) considered it to be due to typical substances occurring in mustard seed oil. However, they demonstrated that this supposition was unlikely.

Carroll et al. ('51, '52, '53) showed that on feeding rapeseed oil the cholesterol content of the adrenals increases owing to the presence of erucic acid. From these experiments it further appeared that rapeseed oil gives a poorer growth

response than other oils investigated at the same time. However, their experiments did not prove that the poor growth of the animals fed rapeseed oil is caused by erucic acid.

The present investigation demonstrates that erucic acid is actually responsible for the growth-inhibiting action of rapeseed oil. The object of the study involved two lines of experimentation. Firstly, the effect on growth of nasturtium seed oil, which contains 80 to 90% of erucic acid, was compared with that of rapeseed oil which contains 40 to 57% of erucic acid (Deuel, '51). Secondly, the effect on growth of mixtures containing erucic acid and tri-erucin respectively, was investigated and compared with that of rapeseed oil.

EXPERIMENTAL

In the investigation 21-day-old male Wistar rats were used. On the basis of their body weight (about 40 gm) the animals were divided into a number of groups, each consisting of 12 animals, and caged separately. In the first part of the experiments 7 groups were used. The nasturtium seed oil was supplied in the diets at the level of 30 Cal. %, while the rapeseed oil was administered at 30, 40, 50, 60, and 73 Cal. % respectively. The 7th group received a diet with 20 Cal. % of summer butterfat, representing the "normal growth" standard. Each food mixture contained 23 Cal. % protein; food and drinking water were available ad libitum. The composition of the diets is shown in table 1.

The diets were freshly prepared twice a week. As usual (Thomasson, '55) the fat-soluble vitamins (A, D, and E) were administered in the form of a prophylactic dose at the beginning of the experiment.

In the second part of the investigation likewise 7 groups of 12 animals each were used. All of the diets contained 20 Cal. % of fat; their composition corresponded with that of the butter group in table 1; only the fat component was different and consisted of:

Group 1: groundnut oil, group 2: groundnut oil fatty acids, group 3: rapeseed oil, group 4: rapeseed

oil fatty acids, group 5: groundnut oil + tri-erucin (50:50), group 6; groundnut oil fatty acids + erucic acid (50:50), group 7: rapeseed oil fatty acids + erucic acid (50:50).

The choice of this experimental design was based on the following consideration. When included in the food at the level of 20 Cal. %, rapeseed oil gives significantly poorer growth than groundnut oil. If erucic acid is actually responsible for the poor growth when rapeseed oil is used, the 50:50 mixture of groundnut oil + tri-erucin should give the

TABLE 1

Composition of the diets

FAT	TEST FAT	RICE	CASEIN	DRIED BREWERS' YEAST	SALT MIXTURE	TEST FAT	CALORIES PER GRAM
	Cal. %	*gm*	*gm*	*gm*	*gm*	*gm*	
Butter fat	20	157.6	34.5	28.5	8.5	21.5	3.99
Rapeseed oil	30	127.4	36.9	28.5	8.5	32.5	4.28
Rapeseed oil	40	97.2	39.4	28.5	8.5	43.0	4.62
Rapeseed oil	50	67.0	41.8	28.5	8.5	53.8	5.01
Rapeseed oil	60	36.8	44.2	28.5	8.5	64.5	5.48
Rapeseed oil	73	...	47.2	28.5	8.5	77.6	6.18
Nasturtium seed oil	30	127.4	36.9	28.5	8.5	32.5	4.28

same growth response as rapeseed oil itself, since both contain about 50% of erucic acid. The same experiment was repeated, but instead of the triglycerides the fatty acids were used. Therefore, in the latter case the reference group received rapeseed oil fatty acids instead of rapeseed oil. The last group was designed to show whether by the addition of erucic acid to rapeseed oil fatty acids the growth would fall below the level of that in the rapeseed-oil groups. This would be a further indication of the growth-inhibiting action of erucic acid. As the unsaponifiable part had been removed from the fatty acids and from the tri-erucin, the experimental scheme described above may give additional information on the influence; if any, of the unsaponifiable fraction of groundnut or rapeseed oil on growth.

The other experimental conditions did not differ from those recorded for the first group of experiments.

RESULTS AND DISCUSSION

The results obtained in the first part of the investigation in which the growth-promoting effect of nasturtium-seed oil was compared with that of rapeseed oil, are given in figure 1.

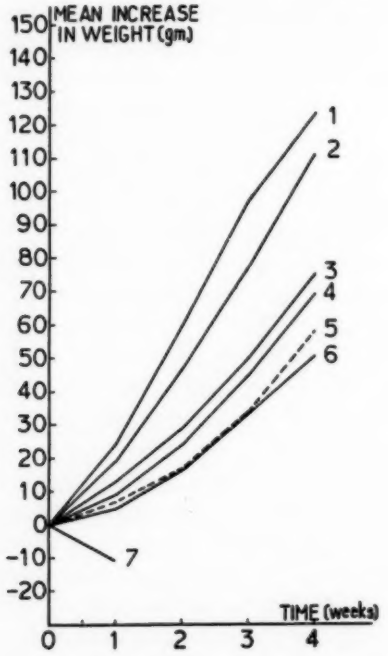

Fig. 1 Growth curves obtained with diets containing butterfat, various amounts of rapeseed oil or nasturtium seed oil.

1: Butterfat, 20 Cal. %
2: Rapeseed oil, 30 Cal. %
3: Rapeseed oil, 40 Cal. %
4: Rapeseed oil, 50 Cal. %
5: Nasturtium seed oil, 30 Cal. %
6: Rapeseed oil, 60 Cal. %
7: Rapeseed oil, 73 Cal. % (11 of the 12 animals died after an average of 16 days).

In agreement with the previous results (Thomasson, '55) 11 out of 12 animals on the diet containing 73 Cal. % of rapeseed oil died. The mean life-span of this group was 16 days; in the other groups no deaths occurred.

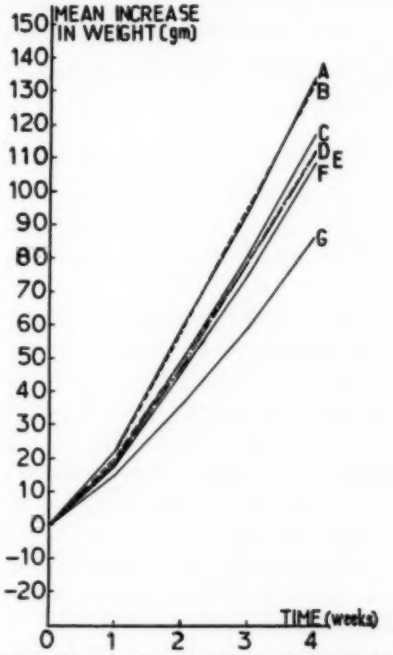

Fig. 2 Growth curves on feeding diets containing 30 Cal. % fat or fat mixtures.
A: Groundnut oil
B: Groundnut oil fatty acids
C: Rapeseed oil fatty acids
D: Groundnut + tri-erucin (50:50)
E: Groundnut fatty acids + erucic acid (50:50)
F: Rapeseed oil
G: Rapeseed oil + erucic acid (50:50)

The results confirm previous observations, namely, that the growth rate of the animals decreases as the rapeseed-oil content of the diets rises. The increase in weight of the rats on a diet containing 30 Cal. % of nasturtium seed oil lies between that of those receiving 50 and 60 Cal. % of rapeseed

oil. By interpolation on the curve it appears that 30 Cal. % of nasturtium-seed oil has the same effect on growth as 57 Cal. % of rapeseed oil. This ratio (1:1.9) is in satisfactory agreement with the erucic-acid contents of the two oils, as a rubber-column analysis [1] (Boldingh, '53) of the nasturtium-seed oil indicated 86% erucic acid. Thus, it has become highly probable that the growth-inhibiting action of rapeseed oil and of nasturtium seed oil is actually due to the presence of erucic acid.

Further proof of this was furnished by the second part of the investigation, in which the animals received tri-erucin and erucic acid, mixed with groundnut oil and groundnut oil fatty acids respectively. The results of this experiment are given in figure 2.

The graph shows that the growth-promoting effect of groundnut or of rapeseed oil does not change when these oils are replaced by their fatty acids. It further appears that growth on the mixture of groundnut-oil and tri-erucin is the same as that when rapeseed oil is fed. The same applies to the corresponding fatty acids, since growth on the mixture of groundnut oil fatty acids and erucic acid was the same as that on the rapeseed oil fatty acids. Moreover it appears that by mixing erucic acid with rapeseed oil fatty acids the growth becomes (significantly) poorer than in the case of the genuine rapeseed oil fatty acids.

These results indicate that the poor growth obtained with rapeseed oil is actually and exclusively due to the presence of erucic acid. In addition, it has been established that under the conditions of the experiment the unsaponifiable fractions of groundnut and rapeseed oil do not affect the growth of the animals.

SUMMARY

Comparison of the growth-promoting effect of nasturtium seed oil, containing 86% of erucic acid, with that of rapeseed

[1] A partition chromatography for fatty acid analysis, in which powdered rubber is used as a carrier for the immobile non-polar liquid phase (light petroleum) in combination with a polar mobile phase (acetone-water).

oil (about 50% erucic acid), as well as determination of the effect of erucic acid on growth, or of fat mixtures containing tri-erucin, has shown that the unfavourable growth obtained with rapeseed oil must be ascribed to the erucic-acid content of this oil.

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NITROGEN METABOLISM AND FAT ABSORPTION IN MALNUTRITION AND IN KWASHIORKOR

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TWO FIGURES

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In two previous papers (Holemans, Lambrechts and Martin, '54a, b) we presented evidence of prolonged severe shortage of proteins in the diets of infants in Kwango (Belgian Congo). As this deficiency is believed to induce kwashiorkor we thought it interesting to study the nitrogen metabolism in children with general malnutrition and kwashiorkor and living in the same region.

For this fundamental study only balance experiments are adequate. As the procedure is difficult to put into practice, only a few experiments are available in the literature. Bray ('53) studied 8 boys and noticed positive balances over long periods of time without gain of weight. More recently, Holmes, Jones and Stanier ('54), made an extensive and careful study of several malnourished African adults. They found a high rate of nitrogen retention continuously for months with a high proportion of fecal nitrogen.

We were interested to know if children suffering from chronic malnutrition suggestive of kwashiorkor but showing the typical signs of that disease were still able to absorb and retain adequate amounts of nitrogen even in desperate cases. Our results show clearly that even a few days before

[1] Fonds Reine Elisabeth d'Assistance Médicale aux Indigènes.

death such cases retain amounts of nitrogen proportional to the intake.

In addition to nitrogen, fat and mineral intakes and losses were investigated. This paper deals only with nitrogen metabolism and fat balances. The literature is confusing about fat excretion in kwashiorkor, most conclusions being based upon either clinical signs (aspect of stools) or percentage of fat in dried specimens of feces. We detected a mild degree of steatorrhea.

METHODS

In Kwango kwashiorkor is not uncommon in infants. Our subjects ranged in weight from 4 to 16 kg. Some babies were only a few months old. It was difficult to ascertain the age of the oldest children which was about 8 or 9 years. Because of technical difficulties and the severity of many cases we limited the study to short balance experiments lasting three days following several days on the experimental diet.

In 29 cases, of which 26 were children and three adults, 44 balances were performed, stools and urine being collected quantitatively by means of a special metabolic cradle for the infants and under continuous supervision for older children.

The children were divided into two groups: those in the first one, "K" (kwashiorkor), showed typical clinical and serological symptoms of kwashiorkor (edema, hair and skin discoloration, low serum albumin, anemia, stunted growth). In the second series "M" (chronic malnutrition), one or more of the above-mentioned signs were lacking except failure to grow. Most of the children had hookworm infestation and received no treatment before the balance experiments. Malarial infection was present in 19 children without fever or splenomegaly. All had anemia with hemoglobin levels from 5 to 11.4 gm per 100 ml. This second series of children, without distinct symptoms of kwashiorkor, were nevertheless severely undernourished and are representative of a large group of Kwango children as reported by Holemans ('54). Fourteen children had alteration of the liver as shown by clinical examination.

We were able to conduct several balance experiments a few days before an autopsy which did not involve histological examination.

Two kinds of diets were given: Diet A contained chiefly animal proteins: fish, meat or milk, skimmed or whole (31 balances). In diet V the protein was derived from vegetable sources: soybeans, peanuts, taken either as vegetables or as "milk" (15 balances). In both groups, the caloric intake varied widely and was not always adequate, varying with the appetite or with a distaste of the patients for food. The individual intakes of nitrogen and calories are shown in the tables. The cutaneous loss of nitrogen was neglected (Holmes et al., '54). We emphasize the fact that the diets used in these studies were not kwashiorkor-inducing but did have a more or less therapeutic value.

In foods, stools and urine, nitrogen was determined by the method of Parnas and Wagner ('21). Proteins were assumed to contain 16% of nitrogen. No tables were used for the composition of foods, a sample of each foodstuff being taken daily and analysed separately. Feces and urine were collected separately and daily analysed for nitrogen and fat. Fat was estimated in food by continuous ether extraction. Milk fat was estimated after Gerber. For the stools, two methods were used: on dried specimens by ether extraction and weighing; on fresh feces by the van de Kamer method ('48). In general, this procedure gave lower values.

RESULTS

A. Nitrogen

The results of our nitrogen balances are recorded in table 1, the groups being listed according to increasing amounts of nitrogen intake. For easy comparison of children very different in weight, the values are expressed in milligrams per kilogram of body weight per 24 hours. Each figure represents the average of three days. The nitrogen intakes differed

TABLE 1

Nitrogen balances

NO. EXP.	WEIGHT	INTAKE	RETENTION	FECAL N	FECAL N CORR.[1]	ABSORPTION	ABSORPTION	CALORIES	DIET	RETENTION	RETENTION
	kg	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*		% [2]	% [2] *corr.*	*kg/24 hr.*		% *intake*	% *absorption*
		(a) Intakes between 100 and 200 mg/kg/24 hr.									
29 K [4]	22	195	113	33	3	83	97	47	A	58	70
32 K	22	173	48	49	19	71	89	43	V	28	39
35 K	22	86	41	37	7	81	93	41	V	48	59
20 M [3]	10	175	87	58	28	67	84	60	A	50	87
24 M	10	158	— 12	82	52	47	67	72	V	..	..
25 M	15.8	184	9	55	25	70	89	45	V	50	71
26 M	15.5	101	— 18	45	15	55	83	56	V	..	..
Average		169	40	51	21	68	86	52		23	34
		(b) Intakes between 200 and 300 mg/kg/24 hr.									
13 K	10	261	113	59	29	77	89	50	A	43	56
15 K	13	233	172	33	3	85	99	31	A	74	87
21 K	8.5	266	140	58	28	78	89	47	V	52	65
22 K	13	214	112	66	36	69	83	44	V	52	75
28 K	12.8	235	78	68	38	71	84	68	A	33	46
30 K	10.4	281	151	50	20	82	93	80	A	54	66
31 K	12.8	290	127	86	56	75	81	70	V	44	59
38 K	38	213	139	43	13	80	94	44	A	65	81
39 K	22	283	117	53	23	82	92	70	V	42	51
6 M	9.3	240	20	43	13	82	94	20	A	8.4	10
37 M	12.8	253	112	70	40	71	84	47	A	44	62
42 K	12.3	204	76	77	47	64	77	48	A	37	68
44 K	10.9	278	153	76	46	73	83	76	A	55	75
45 K	12.3	275	117	105	75	62	73	73	A	42	68
Average		252	123	63	33	75	86.7	55		49	62
		(c) Intakes between 300 and 400 mg/kg/24 hr.									
14 K	30	337	226	19	..	94	..	54	A	70	75
33 K	10.4	372	196	98	68	73	82	94	V	53	74
34 K	12.8	328	194	71	41	82	87.5	71	V	59	72
3 M	13	310	30	67	37	77	88	48	A	9	12
18 [4]	16	343	102	72	42	79	87.4	70	A	30	38
46 M	13.2	377	230	65	35	83	91	110	A	61	74
47 M	10.9	358	240	65	35	82	90	91	A	67	82
Average		346	174	65	35	81	87.5	77		39	48

TABLE 1 *(continued)*

NO. EXP.	WEIGHT	INTAKE	RETENTION	FECAL N	FECAL N CORR.[1]	ABSORPTION	ABSORPTION	CALORIES	DIET	RETENTION	RETENTION
	kg	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*		*%* [2]	*% [3] corr.*	*kg/24 hr.*		*% intake*	*% absorption*
		(d) Intakes between 400 and 500 mg/kg/24 hr.									
10 K	4.4	426	175	137	107	68	75	73	A	41	60
17 K	8.5	496	325	80	50	84	90	127	A	65	77
36 K	10.4	426	331	55	25	87	93	100	V	77	88
41 K	12.4	500	348	92	62	82	87	93	V	70	85
4 M	10	410	160	119	89	70	78	64	A	39	56
19 M	12	444	193	48	18	89	96	90	A	44	49
43 M	13.2	406	226	71	41	83	90	125	A	56	67
Average		444	265	86	56	80	87	96		60	75
		(e) Intakes of 580 mg/kg/24 hr. and more									
2 K	4	690	330	100	70	85	90	100	A	48	56
7 M	4	673	458	94	64	86	90	124	A	68	79
9 K	4.5	929	450	113	83	88	91	158	A	49	56
12 K	4.5	1095	553	70	40	94	96	104	A	49	52
16 K	4.5	1250	496	297	267	75	78	175	A	40	53
40 K	10	647	508	74	34	88	95	108	V	78	89
8 K	4.0	1060	695	335	300	68	71	185	A	65	97
11 M	4.5	1045	440	73	43	92	96	120	A	42	46
1 M	3.7	580	366	42	12	93	98	90	A	63	68
Average		884	477	144	114	85	88	129		54	64

[1] The corrected fecal nitrogen is fecal nitrogen minus 30 mg of metabolic nitrogen.

[2] By percentage absorption of nitrogen is meant: $\frac{\text{intake} - \text{fecal excretion}}{\text{intake}} \times 100.$

[3] The corrected percentage absorption is calculated as follows:

$$\frac{\text{nitrogen intake} - \text{corrected fecal nitrogen}}{\text{nitrogen intake}} \times 100.$$

[4] K refers to kwashiorkor.

[5] M refers to malnourished subjects.

[6] "Normal" child.

widely, partly because of the composition of the diet and partly due to the variations in appetite.

Nitrogen retention. It is clear from the data in table 1 that the nitrogen balances are quite positive. In only two experiments out of 44 was there a negative balance, namely, in the first series, with a low caloric and a low nitrogen intake. In the same experiments a low percentage of nitrogen absorption and a high fecal nitrogen can be observed. In these studies, a negative balance seems to be the exception, due partly to an insufficient intake and partly to impaired digestion. Positive nitrogen balances were also found by Bray ('53), Holmes et al. ('54) in African children and adults and by Karambelkar, Patwardhan and Sreenivasan ('50), Patwardhan, Mukundan, Rama Sastri and Tulpule ('49), Murthy, Swaminathan and Subrahmanyan ('54) for Indian subjects.

We found it unnecessary to record our cases on any other basis than nitrogen intake, because no difference was seen in the results of the balances whether the subject was an infant, a child of 10 years or an adult, whether a typical case of kwashiorkor or an uncomplicated case of undernutrition. The influence of the source of protein on nitrogen metabolism as mentioned in column "diet" will be discussed below.

If the results of all the nitrogen balances irrespective of age, clinical aspect, source of protein and caloric intake are plotted on a graph, it is seen that the nitrogen retention is roughly proportional to the intake (fig. 1).

Optimum daily requirement. Taking the average retention, expressed as percentage of intake, as seen in table 1, this value increases with increasing intake but reaches a maximum of 60% when the mean diet furnishes 444 mg of nitrogen per kilogram of body weight. With a diet higher in nitrogen, greater retention is not obtained. According to Farr ('38), a maximum retention of nitrogen is obtained with an optimum intake of 480 mg of nitrogen per kilogram. This amount represents 3 gm of proteins per kilogram and has been considered by some observers to be the optimum daily requirement in childhood. For 75% of our subjects, even on

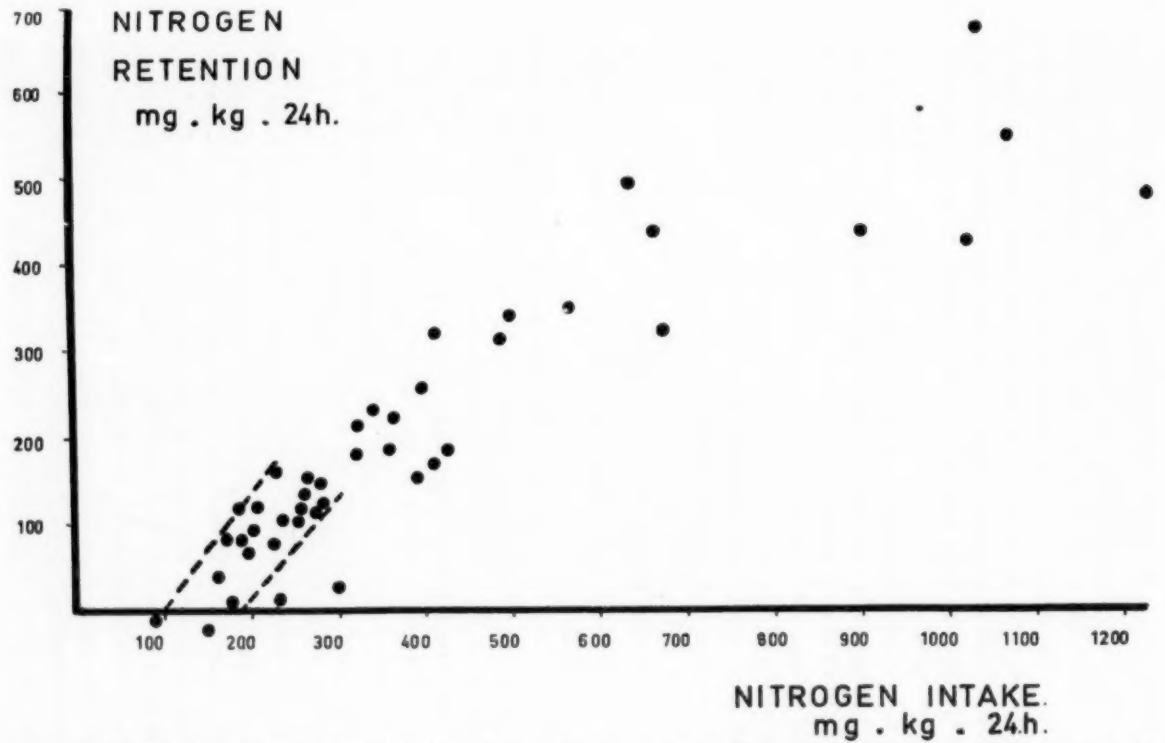

Fig. 1 The relation between nitrogen intake and retention, expressed in milligrams per kilogram per 24 hours. The values are taken from table 1.

hospital diets, the worst of which contained much more nitrogen than the usual diets in the village, the intake was less than our optimum value of about 450 mg. For adults in short experiments on low protein intakes, Murthy et al. ('54) found nitrogen retentions of 54 and 56 mg per kilogram per day on diets which contained respectively 198 and 182 mg per kilogram per day of nitrogen. These results are in keeping with our values.

Minimum daily requirement. The average minimum daily requirement of nitrogen is generally said to lie between 100 and 150 mg per kilogram of body weight (Basu and Basak, '39; Brull, '43) for adults. The abnormal conditions of our balance experiments yield some information about the problem in children. As illustrated in figure 1, the dots representing low nitrogen retention are located in a broad zone between 100 and 200 mg of nitrogen: the zero retention corresponds to an average intake of 100 to 200 mg per kilogram per day. This is the minimum daily requirement of nitrogen established by our experiments.

Percentage absorption. The amount of fecal excretion of nitrogen is an important value in the study of metabolism. Holmes et al. in their extensive research on malnutrition in African adults found that their patients excreted much more nitrogen by way of the bowel than the controls. Their numerous results, expressed in milligrams per gram of dry stools gave an average of about 60 mg in African subjects against 50 mg in the controls.

The column headed "absorption %" in table 1 shows an increasing value (or digestive utilisation) with increasing intake, from 68 to 85%, with an overall average of 79%. Generally the fecal nitrogen is estimated as 10% of the total nitrogen ingested. In other words, the absorption is 90% in adults and in children. In a series of 24 balances for nitrogen and fat absorption in hypotrophic or underweight white infants Lambrechts and Dochain ('55) found an average absorption of nitrogen of 90%. Table 2 summarizes the results.

Fecal nitrogen. Our malnourished subjects compared with European infants on the same intake of nitrogen show a higher excretion of nitrogen in the stools. Their fecal nitrogen is about 20% of the ingested nitrogen, in other words, they waste by the bowel twice as much nitrogen as white children. From table 1, it is obvious that the amount of nitrogen excreted by the bowel is directly related to the quantity of nitrogen of the diet, increasing with rising intake. If the mean values for fecal nitrogen, expressed in milligrams per kilogram of body weight per 24 hours, are plotted against nitrogen intake (fig. 2), a fairly straight line is obtained

TABLE 2

Percentage absorption of nitrogen in white underweight infants
(Liège, Belgium)

AVERAGE INTAKE N	AVERAGE FECAL N	ABSORPTION	ABSORPTION (corrected val.)
mg/kg/24 hr.	*mg/kg/24 hr.*	*% of intake*	*% of intake*
548	50	92	95
653	63	90	95
751	66	91	95
848	71	91	95

(Feshi). For comparison values for underweight white infants (Liège) have been plotted.

By graphical extrapolation (dotted lines) both lines cut the ordinate at the same point corresponding to about 30 mg. This means that on a nitrogen-free diet, 30 mg of nitrogen are still to be found in the stools. This fraction is called metabolic fecal nitrogen (Mitchell, '26). Brull ('43) found in several undernourished adults an average value of 27 mg per kilogram per day. Peters and Van Slyke ('46) say that the average fecal nitrogen of a normal adult on mixed diets is about 1.3 mg daily. This value gives about 20 mg per kilogram of body weight.

Animal and vegetable proteins. As our subjects were on diets composed either of animal proteins (A) or of vegetable

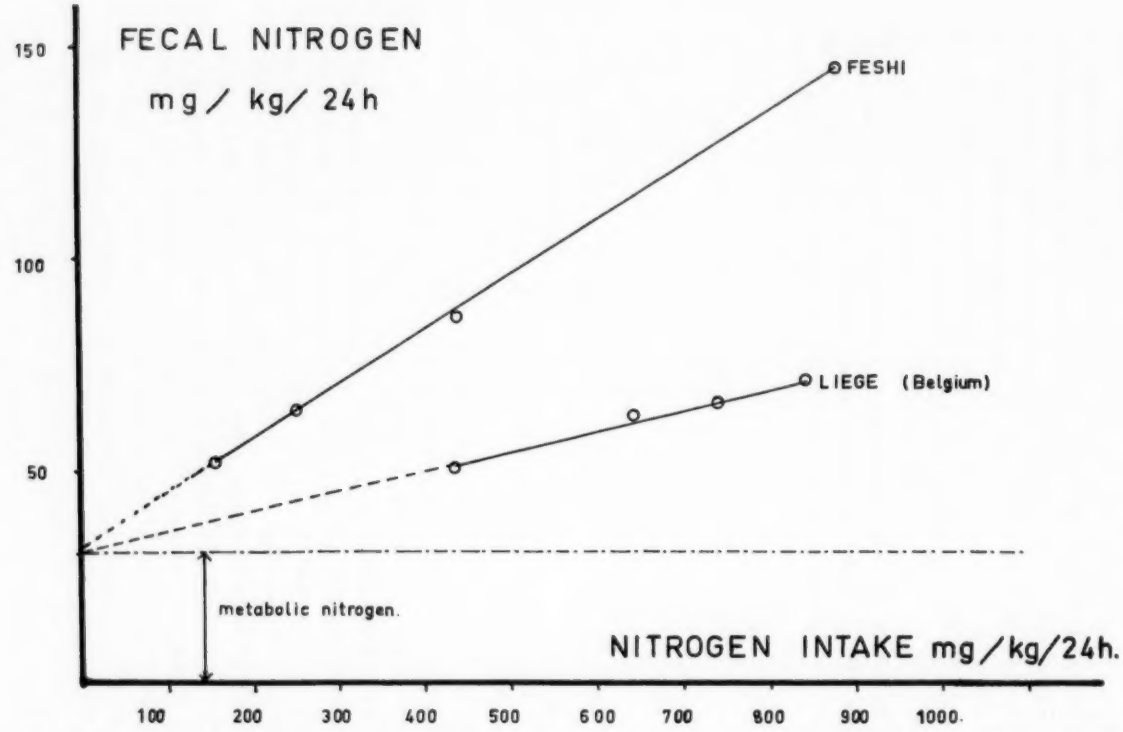

Fig. 2 Relation of fecal nitrogen to nitrogen intake. Upper line gives average values from table 1 (Feshi). Lower line gives average values from 24 balance experiments on hypertrophic infants (Liège), taken from table 2.

proteins (V) it is of interest to compare the absorption and the retention in both cases. The average values are given in table 3 and show that vegetable protein is absorbed and retained as efficiently as animal protein, without significant difference. Karambelkar et al. ('50) and Patwardhan et al.

TABLE 3

Absorption and retention of nitrogen in relation to animal or vegetable proteins in the diet

	DIET A (animal)	DIET V (vegetable)
Absorption, % of intake (corrected value)	85.4	86.4
Retention, % of intake	49	54
Retention, % of corrected absorption	57 ± 5.6	61 ± 4.7

TABLE 4

Possible sparing action of a high caloric diet on nitrogen retention

INTAKE N	CALORIES	RETENTION N
mg/kg/24 hr.		*mg/kg/24 hr.*
426	73	175
426	100	330
410	64	160
406	125	226
690	100	330
673	124	458
1095	104	553
1045	120	440
1060	185	695

('49) found that the retention of nitrogen was higher with vegetable protein than with protein from animal sources.

Possible sparing action. Our malnourished subjects did not furnish any systematic information about the sparing action of high caloric diets on nitrogen metabolism because the nitrogen and caloric intakes varied simultaneously. In a few instances the nitrogen intake remained constant for different caloric values. This is shown in table 4.

B. Fat absorption

Forty-five fat balances were performed on intakes ranging from 0.5 to 5.6 gm per kilogram of body weight per 24 hours (average 1.8 gm). The average percentage of absorption, expressed for fat as for nitrogen, $\frac{\text{Intake} - \text{fecal excretion}}{\text{Intake}} \times 100 =$ 81.8%. This value is lower than normal. No difference was detected between typical kwashiorkor and the other cases of malnutrition. On these levels of intakes normal children have a nearly complete fat absorption of from 90 to 95% of intake according to age (Weyers, '50; Dochain and Lambrechts, '54).

Almost every study dealing with kwashiorkor mentions steatorrhea as an important and quite common symptom in this illness. According to Bergouniou and Tremolieres ('52), fatty stools and diarrhea are common in Dakar but not in other countries. Gillman and Gillman ('51) found in pellagrins with malnutrition more than 30% of fat in the stools of one-half of their cases. Trowell ('37) noticed also a high fat content of the feces. Thompson ('52) stated that excessive fat had been reported in the stools by several observers although no quantitative studies had been given. In Belgian Congo (Kasai), Pieraerts ('49) noticed the regular absence of steatorrhea in his cases; on the other hand Dricot, Beheyt and Charles ('51) concluded that fat digestion was defective, as shown by the presence of microscopic fat in the stools. In our series there was no "clinical" steatorrhea, although sometimes a diarrhea was present but not more frequently than seen in hospitalized children without malnutrition.

We can compare the fat absorption in malnutrition with that in a series of white infants treated for undernutrition (hypotrophy of second grade, 30% underweight) at the pediatric department of the University Hospital of Liège. Detailed results are published elsewhere (Lambrechts and Dochain, '55). In these cases an average fat absorption of 80% was found, a value which became nearer to the normal

level as soon as growth and gain in weight resumed. These patients had an excellent protein digestion as shown before.

Expressed as percentages of dried stools we found in 88 specimens from our subjects in Feshi an average content of 14.4%. This is generally not considered as an abnormal value on mixed diets.

DISCUSSION

It may be difficult to explain satisfactorily the meaning of the positive nitrogen balances in our series. Normal growing children of course retain a very large amount of the ingested nitrogen if the diet is adequate; if the caloric intake is too low, growth is inhibited. Most of our children were stunted in growth, underweight and on an inadequate caloric diet during the experiment: even 100 calories per kilogram of body weight is far below the requirements in underweight or hypotrophic infants. Of course during an acute balance experiment no clinical signs of growth can be detected. But even in the recovery stage of these malnourished children, growth, as evidenced by weight and stature, seems to be the last physiological function to be restored A high nitrogen retention without corresponding gain in weight has been emphasized by Holmes et al. ('54) on adults, Bray ('53) on children, Cook and van Auken ('51), Karambelkar et al. ('50), Patwardhan et al. ('49).

Another explanation of the positive balances in short experiments is the response to a sudden increased intake of protein. It can hardly be said that a diet containing 250 mg of nitrogen per kilogram of body weight per day would produce that condition when the caloric intake is also low. Even in later stages the same children were submitted to repeated balance experiments and behaved in the same way.

A probable explanation is that in these children with ordinary and malignant malnutrition, living since birth on an insufficient nitrogen intake (Holemans, Lambrechts and Martin ('54a, b), the tissues are depleted of proteins. As soon as an amount above the mimimum requirement is given the retention is excellent, about 50% of the intake

It may be emphasized that even in such an abnormal nitrogen economy as in severe malnutrition, the fundamental values of minimum and optimum requirements seem to remain at the same levels as in normal conditions. Retention, as percentage of intake, is likely to be more efficient than in normal individuals. Although the same phenomenon is called "adaptation" by us for calcium and phosphorus metabolism (Holemans, Lambrechts and Martin, '54c) the same cannot be said for nitrogen. Indeed the mineral economy is such that clinical signs of deficiency never become apparent which, of course, is not the case in protein undernutrition.

High fecal nitrogen seems to be a common and regular symptom of malnutrition These features can be detected microscopically (Dricot et al., '51) but better by chemical estimation. We believe it is more desirable to express this value, not as percentage of dry stools, but, either in milligrams per kilogram of body weight per day or as percentage of intake. In this way diet and variable weight are both taken into account and facilitate comparison. The same is true for fat absorption studies where the only convenient expression is not the content per 100 mg of dried stools but the ratio: $\frac{\text{intake} - \text{fecal loss}}{\text{intake}} \times 100$. In this way, in our experiments a mild degree of steatorrhea can be detected. This is an important conclusion which could not have been reached by using the fat contents of dried stools, a value much more likely to vary due to other factors besides fat absorption such as volume of stools and fiber.

Considering simultaneously nitrogen and fat absorption, we may conclude that for both the absorption is lower than normal: twice as much as in normal individuals is lost by the feces. It is well known that in pancreatic diseases fecal nitrogen is increased and according to Thaysen ('26), this is a more reliable procedure for diagnostic purposes than analysis for fat In this way our experiments bring evidence of an impaired digestive function probably of pancreatic origin, which is in keeping with the histological findings of Davies ('48), Waterlow ('48), Vegheliy ('48), Hartz ('49),

Stransky and Dauis Lawas ('50), Manson Bahr ('51), and the biochemical assays of Thompson and Trowell ('52), Danopoulos and Linakardis ('52), Gomez, Calvan and Gravioto ('54), Mukherjee and Werner ('54) in severe malnutrition and kwashiorkor.

Strictly speaking, a high fecal nitrogen and high fat content of the stools can be attributed either to defective digestion or faulty absorption or both. Nevertheless we believe that in our patients with malnutrition and kwashiorkor digestion alone is impaired, and intestinal absorption is normal. The reasons for this assumption are: (1) fecal loss is exaggerated only for those components requiring the intervention of the digestive juices; namely, proteins and fats. Minerals not requiring digestion as calcium and phosphorus, are absorbed normally (Holemans, Lambrechts and Martin, '54c), (2) the metabolic fecal nitrogen estimated by the method of Mitchell ('54) as shown in figures 2 is quite normal; this waste product originates partly in the normal desquamation of the intestinal mucosa.

The fact that vegetable proteins (peanuts, soybeans) are retained equally as well as animal protein is a valuable conclusion in the prophylaxis and treatment of kwashiorkor. Dean ('52) claimed good results in curing this diease by the use of vegetable proteins.

SUMMARY

1. Fourty-four nitrogen and fat balance experiments have been performed in African children living for a long time on protein-deficient diets and suffering from severe malnutrition or kwashiorkor.

2. As a rule, positive nitrogen balances were obtained, showing a retention proportional to the intake (about 50%). Nitrogen was best retained on about 450 mg per kilogram of body weight per day. Even under our experimental conditions the minimum requirements seem to be maintained at the normal level of about 150 mg per kilogram per day.

3. The total fecal nitrogen averaged 21% of the intake; this means a nitrogen absorption of 79%, a lower value than normal. The metabolic fecal nitrogen, however, gave a normal value of 30 mg per kilogram of body weight per day.

4. The nitrogen retention was as good for vegetable proteins as for animal proteins.

5. The fat absorption (80%) is lower than that in normal children (95%).

6. Reasons are given for thinking that only digestion is impaired and that intestinal absorption is normal.

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THE INFLUENCE OF DIETARY FAT ON THIAMINE LOSS FROM THE BODY

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THREE FIGURES

(Received for publication February 24, 1955)

In a recent communication Gershoff and Hegsted ('54) report studies designed to throw light on the mechanism of the thiamine-sparing action of dietary fat. Thiamine-deficient mice showed better survival on a high fat diet; there was, however, no difference in the thiamine concentration in the carcass and liver as compared with animals on a low fat diet. The authors conclude that the rate of thiamine loss from the body is independent of the diet fed, and they criticize the commonly accepted view that since thiamine is required in the metabolism of carbohydrate more thiamine will be required when more carbohydrate is metabolized.

We should like to point out certain features in the experiments of Gershoff and Hegsted which have led them to what we believe is an erroneous conclusion, and to present certain observations of our own made in the human species which indicate that thiamine loss from the body is very definitely influenced by the proportions of the calorigenic foodstuffs given. We therefore see no need to question the accepted explanation for the thiamine-sparing action of fat.

The conclusions of Gershoff and Hegsted in regard to thiamine loss from the body were not based on direct measurements of thiamine excretion, but rather upon deductions from measurements in certain tissues only. They were impressed by the exponential nature of the curves for the depletion of

thiamine in the carcass and liver, but failed to note that the pattern of thiamine depletion is quite different in certain vital tissues which they did not study, notably the brain The accompanying figure (fig. 1) taken from Salcedo et al. ('46) illustrates this difference clearly. The depletion of thiamine in the liver, kidney and heart follow an essentially exponential curve, but the brain holds on to its thiamine without loss

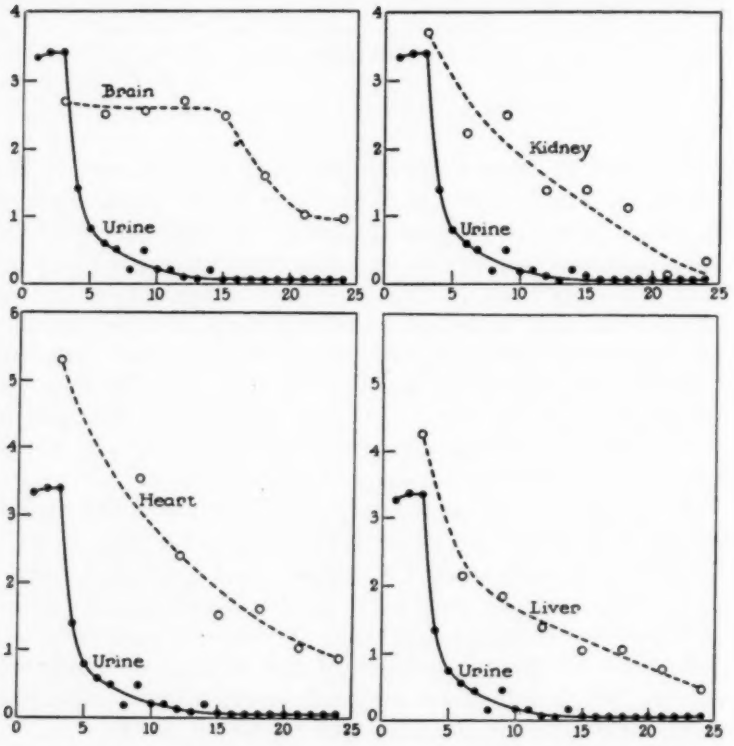

Fig. 1 Relation between thiamine of tissues and thiamine excretion in urine. Ordinates represent micrograms of thiamine per gram dry tissue and micrograms per day of thiamine in urine. Abscissae represent days on thiamine-deficient diet. The tissue figures represent averages of 4 animals sacrificed. The urine figures represent averages of surviving animals. (Taken from Salcedo et al., '48.)

until a critical point is reached, after which it is rapidly lost. Similar observations had been previously reported by Westenbrink ('32). The concentration of thiamine in certain tissues is therefore an unreliable index of what is happening in the body as a whole.

Some years ago in the course of studies to determine the thiamine requirement of infants the present writers undertook some experiments to evaluate the thiamine-sparing effect of fat in the human infant. These experiments, although briefly mentioned in another publication (Holt, '50) have not been published in detail and will now be presented.

EXPERIMENTAL

The first study was carried out on a normal male infant (RDN) of 8 months weighing 8.33 kg. It was planned to evaluate the thiamine-sparing action of fat by following the urinary excretion of thiamine on a rigidly controlled synthetic diet, consisting of vitamin-free casein, hydrogenated cottonseed oil [1], vitamin-free dextrimaltose, a vitamin mixture and a mineral mixture. The details of this diet and the general procedure followed are as given in another publication (Holt et al., '49). The thiamine intake and the proportions of fat and carbohydrate in the diet were altered, the fat and carbohydrate intake being varied over the maximum range commonly employed in infant feeding. The infant was first studied upon an average infant formula providing 15% of the calories as protein, 35% as fat and 50% as carbohydrate. The thiamine intake was adjusted until the thiamine excretion in the urine reached a constant value barely above the minimum level (this was attained at an intake of 0.20 mg of thiamine per day). When equilibrium on this diet had been attained, as judged by the constant urinary thiamine, the formula was changed to a fat-poor carbohydrate-rich diet providing 10% of the calories as protein, 10% as fat and 80% as carbohydrate. As had been anticipated the increase in

[1] Crisco.

carbohydrate calories, presumably by creating an additional demand for thiamine, decreased the urinary thiamine The thiamine intake was then stepped up until the urinary thiamine reached the same level that had been obtained on the original diet. It was found that the caloric shift — essentially a fat to carbohydrate shift — necessitated an increase in the thiamine intake from 0.20 to 0.28 mg per day. The data are presented in figure 2.

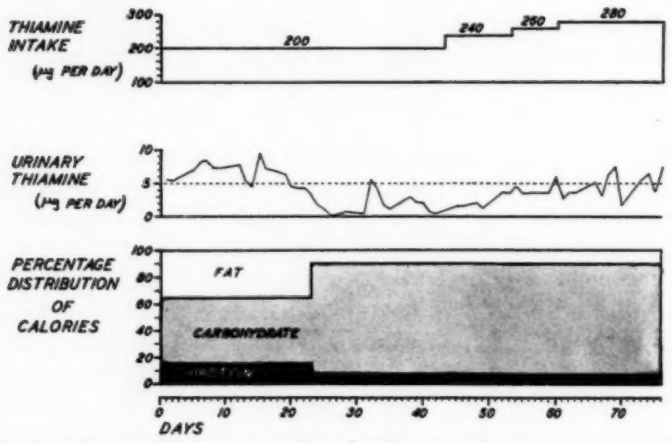

Fig. 2 Relation between composition of diet and thiamine requirement. Subject R.D.N.

A second child (MZ) 18 months old and weighing 8.03 kg was also studied in a similar fashion. He needed 0.24 mg of thiamine to maintain his urinary excretion just above the minimum level when he was given a diet composed of protein 40%, carbohydrate 30% and fat 30%. When the fat was omitted from the diet and the carbohydrate raised to 60%, the thiamine excretion fell off. It was necessary to provide 0.28 mg of thiamine per day to raise these excretion values to their control levels. When 50% of fat was substituted for carbohydrate there was a considerable spill of thiamine into the urine and the thiamine intake could then be returned to 0.24 mg. These data are presented graphically in figure 3.

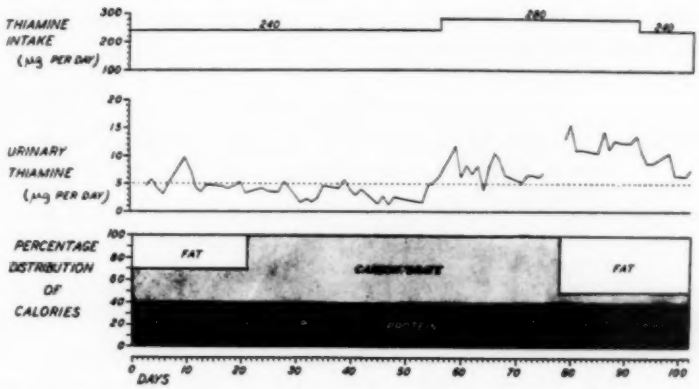

Fig. 3 Relation between composition of diet and thiamine requirement. Subject M.Z.

DISCUSSION

The experiments described show clearly that less thiamine is excreted on a high carbohydrate intake than on a high fat intake, presumably because more is required for metabolic purposes. The results are therefore at variance with the conclusion reached by Gershoff and Hegsted. It is hence unnecessary to attempt to reconcile an increased metabolic demand for thiamine with an unchanged thiamine excretion by postulating that thiamine is not "worn out" by participation in metabolic reactions. The term "worn out" is not a particularly happy one, but the general principle that the participation of a biocatalyst in a reaction involves some degradation of the catalyst itself appears to hold here, even though the nature of this degradation is still far from clear.

SUMMARY

Studies carried out in human infants revealed that the substitution of carbohydrate for fat in the diet resulted in a diminished output of thiamine in the urine. This is interpreted as indicating an increased demand for thiamine in metabolic reactions.

The increased thiamine intake required by such a dietary shift has been measured.

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NICOTINIC ACID METABOLISM IN HUMANS [1]

I. THE URINARY EXCRETION OF NICOTINIC ACID AND ITS METABOLIC DERIVATIVES ON FOUR LEVELS OF DIETARY INTAKE [2]

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The fluorescent derivative of nicotinic acid in urine was first described by Najjar and Wood ('40). The identification of this compound as N^1-Methylnicotinamide (N^1-Me) by Huff and Perlzweig ('43) was followed by the discovery of the oxidized form of this derivative, the 6-pyridone of N^1-Me (pyridone), as a major metabolite of nicotinic acid (Knox and Grossman, '46; '47). The development of methods for the quantitative estimation of these metabolites in urine (Huff and Perlzweig, '47; Rosen et al., '49) made possible more systematic studies on the human metabolism of nicotinic acid. Perlzweig et al. ('50) and Holman and de Lange ('50) reported the average amounts of N^1-Me and pyridone excreted per 24 hours by healthy adult subjects on uncontrolled intakes of the vitamin. Earlier, Ellinger and Kader ('49), using differential analytical techniques, found N^1-Me to be one of the major metabolites of nicotinic acid and nicotinamide in man. Results of studies on the levels of excretion of N^1-Me by adult human subjects on estimated intakes have also been

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reported by Hochberg et al. ('45), Huff and Perlzweig ('47), and Frazier et al. ('49).

Goldsmith and associates ('52) studied the metabolism of nicotinic acid in human subjects on a "corn" diet providing minimum intakes of the vitamin and tryptophan (4.7 and 190 mg, respectively) as well as on a "wheat" diet containing 5.7 mg of nicotinic acid and 230 mg of tryptophan. A more recent study (Rosenthal, Goldsmith and Sarett, '53) reported data on the excretion of the metabolites of nicotinic acid by three normal subjects receiving diets which furnished 10 mg of the vitamin before and after doses of 10, 25 and 50 mg of the amide.

The role of tryptophan as a precursor of nicotinic acid in mammals was established by Heidelberger et al. ('49). The ingestion of tryptophan in excess of dietary levels results in increased excretion of the two major metabolites of nicotinic acid in humans (Perlzweig et al., '47, '50; Sarett and Goldsmith, '47, '50; Holman and de Lange, '50; Sarett, '52; Goldsmith et al., '52; Reddi and Kodicek, '53; Holman, '54).

Studies on the levels of excretion of the metabolic derivatives of nicotinic acid in relation to intakes of the vitamin and its precursor by human subjects maintained on controlled diets should provide more information than is now available on the normal nutrition of humans with respect to this vitamin. In this paper data will be presented on the nicotinic acid metabolism of freshman and sophomore college women maintained on constant diets at 4 levels of dietary nicotinic acid and tryptophan.

EXPERIMENTAL

Subjects

Six healthy young college women, 18 to 20 years of age, served as experimental subjects. The apparent homogeneity of the group is shown by their physical characteristics listed in table 1. One subject (P.T.) was discontinued after 28 days while subjects L. C. and N. L. were off the study during the

adjustment period to the basal diet. L. C. had the measles and N. L. had mild hypermotility of the gastro-intestinal tract. The subjects lived in their usual dormitory environment and participated in the regular activities of the student group.

Plan of study

The subjects ate weighed diets of constant composition for a period of 53 days, which was divided into control 18-, modified control 10-, basal 12-, and post-basal 13-day periods, with intakes of nicotinic acid from dietary sources in order

TABLE 1

The experimental subjects

SUBJECTS	AGE	WEIGHT	HEIGHT	SURFACE AREA [1]	BASAL METABOLISM
	yrs.	*kg*	*cm*	m^2	*Cal./m^2/hr.*
L.C.	18	56.7	163.8	1.62	33.7
S.J.	19	60.2	164.5	1.65	33.5
N.L.	19	66.7	171.5	1.78	35.2
P.T.[2]	18	57.7	160.9	1.69	32.9
J.W.	20	49.3	157.5	1.47	31.4
N.W.	20	59.7	168.9	1.69	30.6
Mean	19	58.4	164.5	1.65	32.9

[1] Calculated by Dubois-Dubois Formula.
[2] Discontinued after 28 days.

of sequence of study periods as follows: control, 11.3 mg; modified control, 14.3 mg; basal, 7.2 mg; and post-basal, 16.3 mg. The metabolic data collected during the control periods of study when the essential nutrients were provided from a variety of dietary sources according to the National Research Council's (NRC) recommended allowances ('53) were used as a basis for comparison with those collected during the basal period.

Experimental diets

The calculated nutritive value of the control diet was as follows: calories, 2135; protein, 70.5 gm (analyzed 73.1);

calcium, 1.13 gm; phosphorus, 1.39 gm; iron, 12.0 mg; vitamin A, 7723 IU; ascorbic acid, 117 mg; thiamine, 1.3 mg; riboflavin, 2.0 mg; and nicotinic acid, 11.8 mg (analyzed 11.3). After 18 days (two study periods) on this diet, the protein and nicotinic acid values were increased to 80 gm and 14.3 mg, respectively (modified control). The basal diet was essentially that described elsewhere (Frazier, '54) with the addition of milk products. The foods included in the basal diet with amounts of each served daily are listed in table 2. This combination of foods furnished a good mixture of the essential amino acids, at the same time providing a minimum intake of nicotinic acid from dietary sources (7.2 mg) and a moderate level of tryptophan. The calculated nutrient content of this diet approached the NRC recommended allowances of other essential nutrients for the group studied. During the post-basal period, the basic pattern of the basal diet was retained, with wheat bread and lean beef replacing the corn bread and hominy or Spanish rice. Peanut butter, milk, and cottage cheese were added in sufficient amounts to bring the protein and nicotinic acid to pre-basal levels. The calorie values of the diets during each of the 4 regimes were approximately the same, average 2,155 exclusive of hard candy. The subjects maintained approximately constant body weights during the 8-week study. The tryptophan content of the control, modified control, basal and post-basal diets was 885, 1,150, 600 and 1,120 mg respectively. The values are based upon assay of diet composites (Frazier, '54) with corrections for changes or additions of food items according to assay of individual foods.

Sampling and analytical methods

After an adjustment period of several days on each dietary regime, 4-day study periods were made weekly. Twenty-four-hour urine collections were made in dark bottles containing 5 ml of toluene and refrigerated at 5°C. A composite containing one-tenth of the urine volume for each of the 4 days

TABLE 2

The basal diet

BREAKFAST		LUNCH		DINNER	
Food	Amount	Food	Amount	Food	Amount
	gm		*gm*		*gm*
Tomato juice[1]	100	Corn bread		Hominy (casserole)[1]	100
Bacon (fried)	10	Corn meal, dry wt.	50	Cream	30
Corn meal mush		Dried milk solids	4.5	Beets, Harvard	60
Corn meal, dry wt.	30	Bacon fat	8	Corn bread (see lunch)	
Oleomargarine	4	Green beans, frozen[1]	60	Salad	
Cream, 18%	20	Bacon fat	5	Cabbage	40
Milk (skimmed)	200	Sweet potatoes	100	Carrots	25
Coffee	150	Pineapple, crushed	36	Cottage cheese	50
Sugar	5	Oleomargarine	8	Olives	10
		Salad		French dressing	14
		Peach halves	100	Apple sauce, canned	100
		Lettuce	15	Tea	150
		Cottage cheese, drained	50	Lemon juice	5
		Mayonnaise	5	Sugar	10
		Ice cream	58	Oleomargarine	8
		Oleomargarine	5		

Hard candy, ad libitum up to 45 gm per day

[1] One hundred grams of orange juice, 70 gm of turnip greens, and Spanish rice casserole were served on alternate days. Spanish rice, ingredients in grams: rice, 20; onion, 9; green peppers, 7; tomatoes, 30; and salad oil, 10.

The calculated nutrients in the diet (averages for the two menus) were as follows: calories, 2083; fat, 83 gm; carbohydrate, 293 gm; calcium, 1.13 gm; phosphorus, 1.59 gm; iron, 9.6 mg; thiamine, 1.08 mg; riboflavin, 1.71 mg; vitamin A, 19200 I.U.; and ascorbic acid, 110 mg. By analysis: 8.2 gm nitrogen and 7.2 mg of nicotinic acid and approximately 600 mg of tryptophan.

was stored in the frozen state until analyzed. Fecal collections were made over the same 4-day period with carmine as a marker. The fecal collections were preserved with H_2SO_4 in a covered contained and held in the frozen state until prepared for analysis.

A one-day diet composite containing weighed servings of all foods eaten except oleomargarine and sugar was prepared for analysis during each of the 4-day study periods. The day's composite was homogenized in a Waring Blendor and a portion of the homogenate was frozen immediately and held until analyzed.

Microbiological procedures were used for the nicotinic acid assay of diet and urine composites (Association of Vitamin Chemists, '51). Quinolinic acid assay was made according to the method of Henderson ('49). N^1-Me was determined by a modification of the method of Huff and Perlzweig ('47) to semi-micro techniques. The fluorescent derivative was measured in a Farrand Fluorophotometer. The method of Rosen et al. ('49), as modified by Sarett and Goldsmith,[5] was used for the assay of the pyridone.[6] Creatinine excretion was determined for completion of collection by the alkaline picrate method. The macro-Kjeldahl method for nitrogen was used for assay of diet, urine, and fecal composites.

RESULTS AND DISCUSSION

Data on the nitrogen balance and the urinary excretion of quinolinic acid, nicotinic acid, N^1-Me, and pyridone for the 6 subjects on each of the 4 dietary regimes are given in table 3. The nicotinic acid values are based upon the results of the assay of untreated urine samples using *lactobacillus arabinosus* as the test organism. According to the results of studies reported by Reddi and Kodicek ('53), who employed chromotographic techniques for the separation of the metabolites of nicotinic acid and nicotinamide, other tertiary

[5] Personal communication.

[6] The pyridone standard was supplied by Sharp and Dohme, Inc., Glenolden, Pennsylvania, through the courtesy of Dr. Jesse W. Huff.

TABLE 3

The nitrogen balance and 24-hour excretion of quinolinic and nicotinic acids, N^1-Me and 6-pyridone on 4 levels of nicotinic acid intake

SUBJECT	NITROGEN BALANCE, NET	QUINOLINIC ACID	NICOTINIC ACID	N^1-Me	PYRIDONE	INTAKE EXCRETED [1]	
						Nicotinic acid equiv.	Ratio Pyridone / N^1-Me
	gm	*mg*	*mg*	*mg*	*mg*	%	
			Control (11.3 mg)				
L.C.	0.56	3.31	0.49	7.10	6.15	102	0.70
S.J.	2.00	3.22	0.52	5.14	5.14	80	0.81
N.L.	1.03	3.59	0.55	7.62	6.59	107	0.70
J.W.	1.20	3.45	0.49	6.25	9.75	118	1.27
N.W.	1.90	2.83	0.53	5.24	8.18	100	1.27
P.T.	0.59	3.25	0.54	5.09	6.79	90	1.08
Mean		3.28	0.52	6.07	7.10	100	0.96
			Modified control (14.3 mg)				
L.C.	0.24	4.26	0.49	7.35	9.36	99	1.04
S.J.	1.51	3.01	0.54	4.61	4.64	57	0.82
N.L.	0.23	3.14	0.56	5.93	7.39	80	1.01
J.W.	0.16	3.67	0.49	6.41	10.16	97	1.29
N.W.	1.42	2.73	0.52	5.44	11.17	96	1.67
P.T.	0.14	2.98	0.55	6.46	8.05	87	1.01
Mean		3.30	0.53	6.03	8.46	86	1.14
			Basal (7.2 mg)				
L.C.	— 0.59	3.55	0.45	(6.14)	(7.52)	161	0.99
S.J.	0.97	2.71	0.42	2.69	4.87	90	1.47
N.L.	— 1.23	2.67	0.45	(5.33)	(6.76)	143	1.03
J.W.	0.54	3.44	0.41	3.18	8.77	136	2.25
N.W.	0.51	2.41	0.49	2.80	6.75	112	1.96
Mean		2.96	0.44	2.89 [2]	6.80 [2]	128	1.40
			Post-basal (16.3 mg)				
L.C.	2.02	2.85	0.50	4.27	7.33	60	1.42
S.J.	3.90	3.12	0.62	5.64	8.17	72	1.18
N.L.	1.37	2.95	0.58	6.75	9.28	83	1.12
J.W.	1.57	3.72	0.49	5.26	12.01	87	1.86
N.W.	3.10	3.25	0.52	5.42	11.14	84	1.67
Mean		3.18	0.54	5.47	9.59	77	1.44

[1] Expressed as nicotinic acid.

[2] Values in ( ) for L.C. and N.L. not included in average.

pyridine compounds are not normal metabolites of the vitamin. The values for quinolinic acid were derived from differences in the amounts of nicotinic acid found by microbiological assay before and after treatment of urine samples with glacial acetic acid (Henderson, '49).

The absolute amounts of nicotinic acid excreted as the vitamin and the two methylated derivatives (expressed as nicotinic acid) averaged 11.30, 12.28, 9.24 and 12.60 mg for the 4 dietary regimes, namely control, modified control, basal, and post-basal, respectively.

An increase of 3 mg of dietary nicotinic acid in the modified control diet over that in the control diet had no effect upon the average amounts of the vitamin and N^1-Me excreted per 24 hours by the 6 subjects. These values on the control and modified control diets, respectively, were: nicotinic acid, 0.52 and 0.53 mg; and N^1-Me, 6.07 and 6.03 mg. The average excretion of pyridone increased from 7.10 to 8.46 mg per day. After 6 days on the basal diet with a decrease in intake of 7 mg of nicotinic acid (from 14.3 to 7.2 mg), three of the 6 subjects excreted an average of 2.89 mg of N^1-Me and 6.80 mg of pyridone per 24 hours. With the change to a more liberal diet during the post-basal period, an increase in nicotinic acid of 9 mg per day (from 7.2 to 16.3 mg), and a return to control levels of dietary protein and tryptophan, the average amounts of nicotinic acid and N^1-Me excreted returned to approximately pre-basal levels. The increase in the excretion of pyridone, on the other hand, exceeded the control level by an average of 2.49 mg per day. The average increase over the modified control level was 1.13 mg.

Thirty-three per cent of the 3 mg difference in the two control diets was found in the urine in the form of pyridone. Likewise, 37% of the 9 mg increase (from basal to post-basal) was reflected in the amounts of nicotinic acid and its two major metabolites found in the urine. With a decrease in intake from 14.3 to 7.2 mg, there was a mean decrease of 43% of the 7 mg decrease in intake. The order of increase in the excretion of N^1-Me and pyridone with increased intakes

of dietary nicotinic acid and tryptophan is similar to that found by Goldsmith et al. ('52). These investigators observed a larger increase in the quantity of the oxidized derivative excreted after ingestion of the two compounds. The consistency in the relation of relatively small increases in dietary nicotinic acid to the increase in excretion seems to indicate that uniform adjustment occurs in the metabolism of this vitamin and that approximately one-third of the increase is excreted as the pyridone.

Although information on the percentage of the dietary nicotinic acid excreted in the urine does not account for that contributed by the precursor or body stores, it does provide an indication of the total amount of the vitamin metabolized. In the present study the 6 women excreted an average of approximately 100% of the 11.3 mg in the control diet. On the basal diet 128% of the intake was excreted as the vitamin and its two major excretory products, while 86 and 77%, respectively, of the intakes of 14.3 and 16.3 mg were found in the urine. Leifer et al. ('51), as well as Lin and Johnson ('53), found $C^{14}O_2$ in the expired air of rats after ingestion of the radioactive forms of the vitamin. The amount of the radioactive carbon in the expired air accounted for approximately 15% of that ingested.

Perlzweig, Rosen, and Pearson ('50) reported that the ratio of pyridine to N^1-Me excreted by 10 healthy adults on self-selected diets averaged 1.37 (range 1.0 to 1.8). The average ratio of the oxidized form to N^1-Me (calculated to the equivalent amounts of nicotinic acid) in the present study was approximately one or above on the 4 levels of dietary nicotinic acid. The average ratios for the 6 subjects increased with the amount ingested in the two control and post-basal diets in the following order: $11.3 < 14.3 < 16.3$ mg. These data are in agreement with those reported by Rosenthal, Goldsmith, and Sarett ('53), who found a relatively constant amount of N^1-Me excreted by normal subjects on 10 mg of nicotinic acid before and after a supplement of 10 mg of nicotinamide. After 25- and 50-mg test doses of the vitamin,

there was some increase in N^1Me but not in proportion to that of pyridone, which showed significant increases at all levels of the supplement from 10 to 50 mg. These results suggested to the investigators (Rosenthal et al., '53) that ingestion of the vitamin in excess of that required for normal body processes is disposed of as the oxidized derivative.

The three subjects (S. J., J. W. and N. W.) who remained on the constant diets during the entire 53-day period either approached nitrogen equilibrium or stored nitrogen on the 4 protein intake levels; namely, 73, 80, 51, and 80 gm per day during the control, modified control, basal, and post-basal periods, respectively. Subjects L. C. and N. L., who did not observe the basal adjustment period, were in negative nitrogen balance during the basal period of study.

In general, the patterns of excretion of quinolinic acid and the two major excretory products of nicotinic acid for each subject were similar. However, there was considerable variation in metabolic patterns among subjects. For example, J. W. and N. W. consistently excreted larger amounts of pyridone than the other three subjects. S. J., on the other hand, showed a lower level of excretion at all levels of intake (table 3). This subject (18 years) had a history of amenorrhea and did not menstruate during the entire 8-week period. The metabolic performance of L. C. and N. L. is of interest in view of the fact that the study of these two subjects was interrupted for reasons already stated. The data reported for these subjects on the basal diet represent the amounts of the metabolites found in the urine during days one to 4 on the restricted intake, whereas those for S. J., J. W., and N. W. were the average amounts excreted per 24 hours from days 7 to 10, inclusive. L. C. ate family meals during the time she was off the study, while N. L. ate in the college dining hall and consumed at least one quart of milk daily upon recommendation of the college physician. The difference in the data on the excretion of N^1-Me and pyridone for these two subjects, as compared with the other three girls, probably reflects the influence of body stores of the vitamin and its

precursor and emphasizes the importance of establishing biochemical equilibrium in the quantitative study of nicotinic acid nutrition. N. L. excreted decreasing amounts of quinolinic acid from control period 1 through post-basal period 5. It was not until the last 4-day study period that the excretion of the tryptophan derivative returned to the initial level. At the same time she went into slight negative nitrogen balance. This subject, who was 19 years old and weighed 67 kg, showed evidence of hypergastro-intestinal motility throughout the study. She absorbed only 90% of the dietary protein on the control and post-basal diets as compared with J. W., for example, who absorbed 94% of the protein ingested. J. W. was 20 years of age and weighed 49 kg. Therefore, it seems possible that the variation in the metabolic pattern with respect to nitrogen and the tryptophan derivative of this subject (N. L.), as compared with the other subjects, may have resulted from insufficient protein, especially during the basal period.

In this group of apparently homogeneous subjects, certain variations in over-all metabolism were observed that may have an influence on the availability and utilization of the nutrients studied. For example, the mild hypermotility of the G. I. tract of the subject N. L. was considered normal for this individual by the consulting physician. In the case of J. W. and N. W., on the other hand, there was a delay of two to 4 days in the excretion of the carmine both at the beginning and end of the fecal collection period. These two subjects absorbed a higher percentage of the dietary protein and showed a higher net nitrogen balance (table 3). It has been pointed out that the levels of excretion of pyridone by these two subjects were consistently higher than those observed for the others. Since the excretion of N^1-Me varied among subjects within a relatively narrow range, the ratio of pyridone to N^1-Me for J. W. and N. W. was higher at all levels of intake.

There are no data available on the rate of conversion of tryptophan in humans on "normal" levels of intake of the

two nutrients. Schayer and Henderson ('52) have shown that in the rat the nitrogen of the indole ring of tryptophan is probably the only source of the nitrogen of quinolinic acid. Although the quantity of quinolinic acid found in the urine varied with dietary tryptophan, the absolute amount of this metabolite excreted by individual subjects varied within a narrow range between study periods. At the present time it is not clear whether quinolinic acid is in the direct path of the interconversion of the amino acid to the vitamin or whether it is the product of a side reaction. Holman ('54) found only a slight increase in the amounts of N^1-Me and pyridone excreted by three healthy subjects after an oral dose of 300 mg of quinolinic acid, as well as after intravenous injection of this amount. In this connection attention is called to the relation of the quantity of quinolinic acid excreted per 24 hours to the dietary tryptophan. On the control diet (885 mg of tryptophan) the percentage of tryptophan equivalents excreted as quinolinic acid by the subjects L. C., S. J., N. L., J. W., N. W., and P. T. were 0.46, 0.44, 0.49, 0.47, 0.39, and 0.45, respectively. The tryptophan intakes, except in the case of the control period are approximate only. The value of 885 mg is based upon analysis of diet composites, while the other three values are estimated as described under procedure. Test doses of 100 mg of L-tryptophan were given S. J., J. W., and N. W. immediately following the basal study period, and the amount of quinolinic acid excreted in the following 24 hours as compared with the preceding 24-hour control period was measured. The increase in the excretion of this metabolite amounted to 0.45, 0.46, and 0.58% of the ingested tryptophan for the three subjects, respectively.

Goldsmith and her associates ('52) confirmed the estimate of Frazier and Friedemann ('46) that approximately 7.5 mg of nicotinic acid is the minimum daily requirement on marginal diets. Since the interconversion of tryptophan to nicotinic acid seems to be a normal route of metabolism for the amino acid, the assessment of the human requirement of dietary nicotinic acid must await more specific information on the

rate of conversion of tryptophan to nicotinic acid under specific dietary conditions. However, in normal nutrition an amount of the vitamin in excess of that excreted seems to be desirable for girls in late adolescence. In the present study the 5 young women on the basal "corn" diet, providing 7.2 mg of nicotinic acid and 600 mg of tryptophan, excreted an excess of approximately 30% of the amount ingested, indicating that approximately one-third (2.04 mg) of the 9.24 mg excreted as the vitamin and its two major metabolites was supplied from the precursor and body stores or both. Moreover, on the control diet containing approximately the recommended allowance, 11.3 mg of nicotinic acid, the total average excretion (11.30 mg) balanced intake. In view of the variability in the metabolic patterns shown among this group of subjects, an intake of 14.3 to 16.3 mg of dietary nicotinic acid per day seems to provide a margin of safety in the over-all requirement of the age and sex group studied under the experimental conditions imposed.

SUMMARY

The nicotinic acid metabolism of 6 freshman and sophomore college women on 4 levels of dietary intake of nicotinic acid and tryptophan was studied.

On a control diet providing 11.3 mg of the vitamin and 885 mg of tryptophan, the average amount excreted by the 6 subjects as the vitamin and its two major metabolites, N^1-Me and pyridone, balanced the intake. When the dietary nicotinic acid of the control diet was increased to 14.3 mg with a concomitant increase in tryptophan, there was a slight increase (1.0 mg) in the total amount of the vitamin and its metabolic derivatives found in the urine. Following the 28-day control periods of study, a basal diet containing 7.2 mg of nicotinic acid and 600 mg of tryptophan was fed for 11 days. The average decrease in total excretion during this regime as compared with the immediately preceding control level of excretion was 3.04 mg. On the basal diet the total average excretion (expressed as nicotinic acid) was 128% of the

amount ingested. During the post-basal period with an increase to 16.3 mg intake, the excretion of nicotinic acid and N^1-Me returned to control levels, while the amount of pyridone exceeded the pre-basal level.

With each increment in dietary nicotinic acid approximately one-third of the increase was excreted as pyridon; on the other hand, a comparable decrease in intake of the vitamin was reflected in decreased amounts of the vitamin and N^1Me excreted as well as the pyridone.

There was considerable variation in the metabolic performance among the 6 experimental subjects.

The significance of the findings in this study has been discussed in relation to "normal" nutrition with respect to the nutrients studied.

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FACTORS INFLUENCING ADRENAL WEIGHT AND ADRENAL CHOLESTEROL IN RATS FOLLOWING STRESS[1]

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FOUR FIGURES

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INTRODUCTION

Adrenal weight and adrenal cholesterol concentration are among the most widely used indices of adrenal function in experimental animals (Long, 47; Selye, '50). After an acute stress such as the injection of adrenocorticotropic hormone (ACTH), the adrenal cholesterol of a normal rat is depleted within three to 6 hours, and is restored within 24 hours (Sayers et al., '44). Although experiments have shown that resting adrenal cholesterol levels can be modified by diet (Carroll, '51; Winters et al., '52; Morgan and Lewis, '52), the effect of dietary constituents on the response of the adrenal to stress has been less extensively investigated. Experiments reported previously showed that the restoration of adrenal cholesterol after stress was delayed in rats on diets deficient in pantothenate (Dumm et al., '53). In the present study, the effect of large amounts of calcium pantothenate, ascorbic acid, and of 16 and 22% protein diets on adrenal cholesterol and adrenal weight following stress were determined. In addition, the response of male rats has been compared with that of female rats.

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PROCEDURES

All rats used in the experiments were bred in the laboratory. They were started on an experimental diet at about 45 days of age and continued on the same diet throughout the experiment. A total of 391 rats were studied. The number in each diet group is shown in table 2.

After at least 4 weeks on an experimental diet, the animals were subjected to surgical stress. Two types of stress were used: (1) unilateral adrenalectomy alone, and (2) unilateral adrenalectomy plus skin denudation. In the latter, an amount of skin equal to 4% of the body weight, which is approximately 15% of the surface area, was removed from the animal's back (Ralli et al., '54). At the time of the initial stress the left adrenal was removed, weighed and analyzed for its cholesterol content. After varying intervals, the right adrenal was removed and the analyses repeated. The weight and cholesterol content of the right adrenal were compared to the weight and cholesterol content of the left adrenal of the same rat.

The composition of the diets is summarized in table 1. Five diets were used in the unilateral adrenalectomy experiments (Diets A, B, C, D, and G). Diet A contained 22% protein, and more than adequate amounts of all vitamins essential for rats (Brown and Sturtevant, '49). Diet B (22% protein, high pantothenate) was identical to Diet A except for the inclusion of 43.6 mg of calcium pantothenate per 100 gm of diet. Diet G (22% protein, high ascorbate) was identical to Diet A except for the addition of 1 gm of ascorbic acid per 100 gm of diet. Diets C and D contained 16% protein. Diet C contained calcium pantothenate in the amount required by rats from 5 to 7 weeks of age (Unna and Richards, '42). Diet D (16% protein, high pantothenate) contained 43.6 mg of calcium pantothenate per 100 gm of diet and was otherwise identical to Diet C. Rats on the 16% protein diets (C and D) were continued on the diets for 7 to 8 weeks before unilateral adrenalectomy and were approximately 90 days of age at the time of operation. Rats on the 22% protein diets (Diets

A, B and G) received the diets for 4 weeks before unilateral adrenalectomy, at which time they were about 65 days of age. All animals used in the unilateral adrenalectomy experiments received a 1% solution of sodium chloride as drinking water.

Diets E and F, used in the experiments on skin denudation plus unilateral adrenalectomy, also contained 22% protein and either adequate (Diet D) or large amounts of panto-

TABLE 1

Composition of experimental diets

(Quantities for 100 gm of diet)

CONSTITUENTS	DIET A	DIET B	DIET C	DIET D	DIET E	DIET F	DIET G
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Vitamin free casein	22	22	16	16	22	22	22
Sucrose	62	62	68	68	64	64	64
Hydrogenated cottonseed oil	9	9	9	9	9	9	9
Cod liver oil	2	2	2	2	2	2	2
Salt mixture A	5	5	5	5	0	0	5
Salt mixture B	0	0	0	0	3	3	0
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Thiamine	0.30	0.30	0.15	0.15	0.30	0.30	0.30
Pyridoxine	0.30	0.30	0.15	0.15	0.30	0.30	0.30
Riboflavin	0.50	0.50	0.25	0.25	0.50	0.50	0.50
Folic acid	0.10	0.10	0.05	0.05	0.10	0.10	0.10
Biotin	0.05	0.05	0.025	0.025	0.05	0.05	0.05
Calcium pantothenate	1.0	43.6	0.50	43.6	1.0	43.6	1.0
Niacin	1.0	1.0	0.50	0.50	1.0	1.0	1.0
Choline	30	30	30	30	30	30	30
Inositol	5.0	5.0	2.5	2.5	5.0	5.0	5.0
Mixed tocopherols	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Vitamin K	5.0	5.0	2.5	2.5	5.0	5.0	5.0
Ascorbic acid	0.0	0.0	0.0	0.0	0.0	0.0	1000

One hundred grams Salt Mixture A: 2 gm ferric citrate, 44 gm calcium phosphate (dibasic), 42 gm potassium citrate, 12 gm magnesium citrate, 80 mg copper sulfate, 10 mg potassium iodide.

Salt Mixture B was identical with Salt Mixture A except for the omission of the potassium citrate.

Drinking solution with Diets A, B, E and F — 1% NaCl.

Drinking solution with Diets C and D — 0.100 M NaCl plus 0.050 M KCl.

thenate (Diet F). Diets E and F differed from Diets A and B in that no potassium was included. The drinking solution used with Diets E and F contained both NaCl (0.100 M) and KCl (0.050 M) and was available ad libitum.

RESULTS

Adrenal weight and cholesterol prior to the stress

Table 2 summarizes the body weight, the absolute weight of one adrenal, adrenal cholesterol concentration, and the total adrenal cholesterol of the rats at the time of operation. The male rats were heavier and had smaller adrenals than did the female rats of the same age and on the same diet. The ingestion of either additional calcium pantothenate or of ascorbic acid was without significant effect on the weight or cholesterol content of the adrenals of the unstressed animals (Diets B and G compared to A). In male rats on the 16% protein diet (Diet C), the adrenal cholesterol was significantly higher than on the 22% protein diet (Diet A). In the female rats, the protein content of the diet did not affect adrenal cholesterol concentration prior to the stress.

Growth from the start of the experiment to the time of the stress was slower on the 16 than on the 22% protein diets in both male and female rats. Within any one diet group there was no relation between adrenal and body weight in either male or female animals of the age group studied in these experiments.

Effect of calcium pantothenate or ascorbic acid on adrenal changes after stress

The effect of large amounts of calcium pantothenate or of ascorbic acid on the rate of restoration of adrenal cholesterol following unilateral adrenalectomy is presented in figure 1. Each point represents the average percentage change for 6 or more rats from the time of operation to the time of sacrifice. The decrease in adrenal cholesterol concentration at 6 hours was greater in the control rats (Diet A) than in

TABLE 2

Effect of diet on adrenal and body weight and on adrenal cholesterol

DIET	% PROTEIN	PANTOTHENATE	ASCORBIC ACID	SEX	NO. OF RATS	BODY WEIGHT		WEIGHT OF LEFT ADRENAL		ADRENAL CHOLESTEROL CONCENTRATION		CHOLESTEROL CONTENT OF LEFT ADRENAL	
						Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
		mg/100 gm	*mg/100 gm*			*gm*	*gm*	*mg*	*mg*	*gm %*	*gm %*	*mg*	*mg*
A	22	1.0	0	M	41	208	38	15.8	3.6	4.47	1.17	.736	.170
B	22	40	0	M	37	216	33	16.6	2.9	4.37	0.86	.719	.151
G	22	1.0	1000	M	42	193	32	16.6	2.5	4.72	0.90	.778	.151
C	16	0.5	0	M	32	223	19	16.0	3.0	5.63 [2]	1.13	.904 [2]	.235
D	16	40	0	M	37	238 [1]	32	16.2	2.3	5.32 [2]	1.19	.867 [2]	.257
A	22	1.0	0	F	44	157	14	22.9 [2]	3.3	5.23 [2]	0.82	1.170 [2]	.214
C	16	0.5	0	F	33	165	25	20.7 [2]	3.7	5.03	0.95	1.035 [2]	.269
D	16	40	0	F	35	175	27	20.2 [2]	5.3	5.13	1.09	1.039 [2]	.266
E	22	1.0	0	M	48	201	37	16.9	2.9	4.51	0.89	.766	.188
F	22	40	0	M	42	198	56	16.7	3.1	4.48	1.04	.739	.204

[1] Difference significant at 0.05 level: Body weight of males on Diet D to males on Diet C.

[2] Differences significant at 0.01 level: Adrenal weight of females on Diet A to males on Diet A, of females on Diet C to males on Diet C, of females on Diet D to males on Diet D; Adrenal cholesterol concentration of males on Diet C to males on Diet A, of males on Diet D to males on Diet B, of females on Diet A to males on Diet A; Cholesterol content of left adrenal to males on Diet C to males on Diet A, of males on Diet D to males on Diet B, of females on Diet A to males on Diet A, of females on Diet C to males on Diet C and of females on Diet D to males on Diet D.

those on the high pantothenate (Diet B) or high ascorbic acid (Diet G) diets. From 5 days on, there was little difference in adrenal cholesterol concentration between the control and high pantothenate rats. In animals receiving large amounts of ascorbic acid (Diet G), the restoration of adrenal choles-

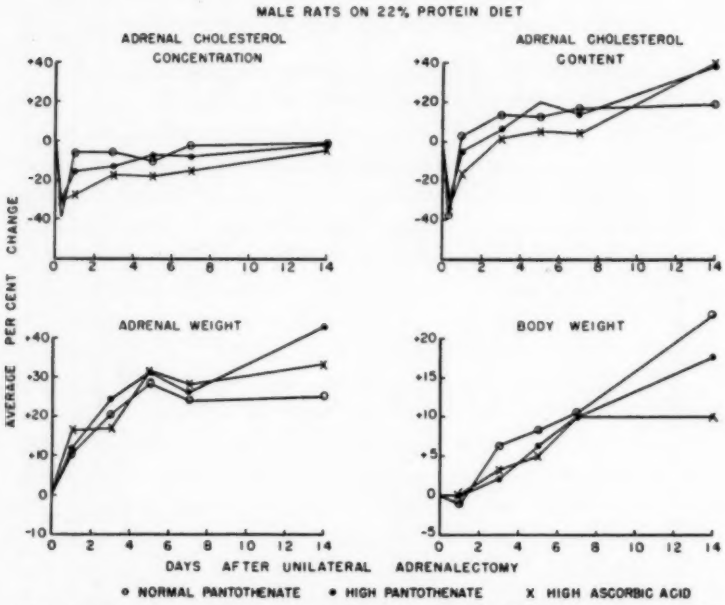
MALE RATS ON 22% PROTEIN DIET
ADRENAL CHOLESTEROL CONCENTRATION
ADRENAL CHOLESTEROL CONTENT
ADRENAL WEIGHT
BODY WEIGHT
AVERAGE PER CENT CHANGE
DAYS AFTER UNILATERAL ADRENALECTOMY
o NORMAL PANTOTHENATE • HIGH PANTOTHENATE X HIGH ASCORBIC ACID

Fig. 1 The average per cent change in adrenal cholesterol concentration, adrenal cholesterol content, adrenal weight and body weight is shown for male rats on a 22% protein diet containing a normal amount of pantothenate (o–o), a very large amount of pantothenate (•–•), or a very large amount of ascorbic acid (x–x). Each point represents the average change in 6 or more rats from 6 hours to 14 days after unilateral adrenalectomy to the time of sacrifice.

terol was significantly retarded up to 7 days after unilateral adrenalectomy. By 14 days, adrenal cholesterol concentration had returned to approximately its original value in all groups of animals. Five days after unilateral adrenalectomy, adrenal weight had increased about 30% in all groups of rats. By 14 days the increase in adrenal weight was 33% in the

rats treated with ascorbic acid, 43% in those treated with calcium pantothenate and 25% in the control animals.

Effect of the protein content of a normal pantothenate diet on adrenal changes after stress

In figure 2, the effect of the protein content of the diet on the rate of restoration of adrenal cholesterol by male rats following stress is shown. The animals received either the 16 or the 22% protein diet (Diets C and A). Six hours after unilateral adrenalectomy, adrenal cholesterol decreased 45% in the rats on the 16% protein diet and 39% in the rats on the 22% protein diet. At 24 hours, the concentration of adrenal

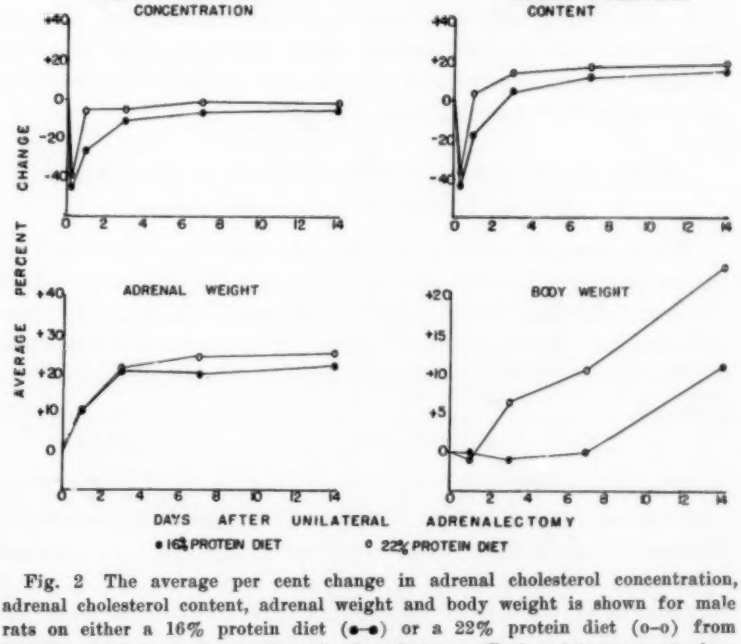

Fig. 2 The average per cent change in adrenal cholesterol concentration, adrenal cholesterol content, adrenal weight and body weight is shown for male rats on either a 16% protein diet (•–•) or a 22% protein diet (o–o) from 6 hours to 14 days after unilateral adrenalectomy. Each point represents 6 or more rats.

cholesterol was 6% below the pre-stress level in the rats on the 22% protein diet, but was 26% below the pre-stress level in the rats on the 16% protein diet. Adrenal weight responses were identical for the first three days after unilateral adrenalectomy regardless of the protein content of the diet and remained very nearly the same throughout the course of the experiment. There was a marked difference in the weight gain of animals on the two levels of protein intake. On the 22% protein diet there was a steady gain in weight from the third day after unilateral adrenalectomy. At 14 days the increase was 25% above the weight at operation. On the 16% protein diet the rats lost weight during the first 4 days after unilateral adrenalectomy. At 7 days their body weight was about the same as at the time of operation and by 14 days the weight had increased 11% above the pre-stress value.

In the female rats after unilateral adrenalectomy, the protein content of the diet had an effect similar to that observed in males on the restoration of adrenal cholesterol concentration and on the gain in body weight.

Effect of the protein content of the high pantothenate diet on adrenal changes after stress

When animals on a high pantothenate diet were fed the same two levels of protein (Diets B and D), the gain in body weight following stress was greater on the high (22%) than on the low (16%) protein intake. There was no significant difference in the restoration of adrenal cholesterol or adrenal weight.

Effect of sex on adrenal changes after stress

The effect of sex on the adrenal response to the stress of unilateral adrenalectomy is shown in figure 3. Data are presented on male and female rats on a 22% protein adequate pantothenate diet (Diet A). In the female animals, the decrease in adrenal cholesterol after the stress was more prolonged than in male animals. Adrenal cholesterol concentration in female rats did not return to its original value

until approximately 7 days after unilateral adrenalectomy. By 14 days after the stress, the percentage change in total cholesterol per adrenal was similar in male and female animals. The response in adrenal weight after the stress was similar in male compared to female rats. The restoration of adrenal cholesterol was also slower in female than in

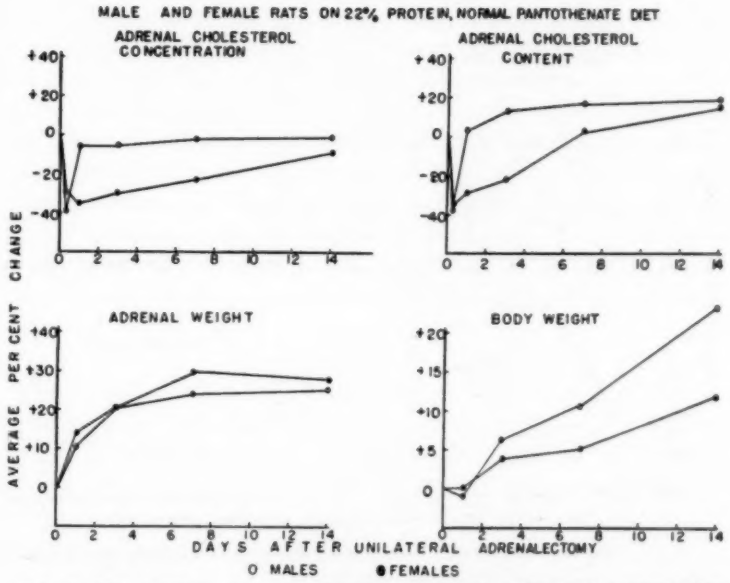

Fig. 3 The average per cent change in adrenal cholesterol concentration, adrenal cholesterol content, adrenal weight and body weight is shown for male (o–o) and female (●–●) rats on a 22% normal pantothenate diet, from 6 hours to 14 days after unilateral adrenalectomy. Each point represents 6 or more rats.

male rats on the 16% protein diet regardless of the pantothenate supplementation (Diets C and D).

Effect of skin denudation plus unilateral adrenalectomy on adrenal changes

The effects of unilateral adrenalectomy plus skin denudation on body and adrenal weight and on adrenal cholesterol for

male rats on the 22% protein diet are presented in figure 4. In one group the diet was adequate in pantothenate (Diet E) and in the other group the diet was high in pantothenate (Diet F). About two-thirds of the rats survived the com-

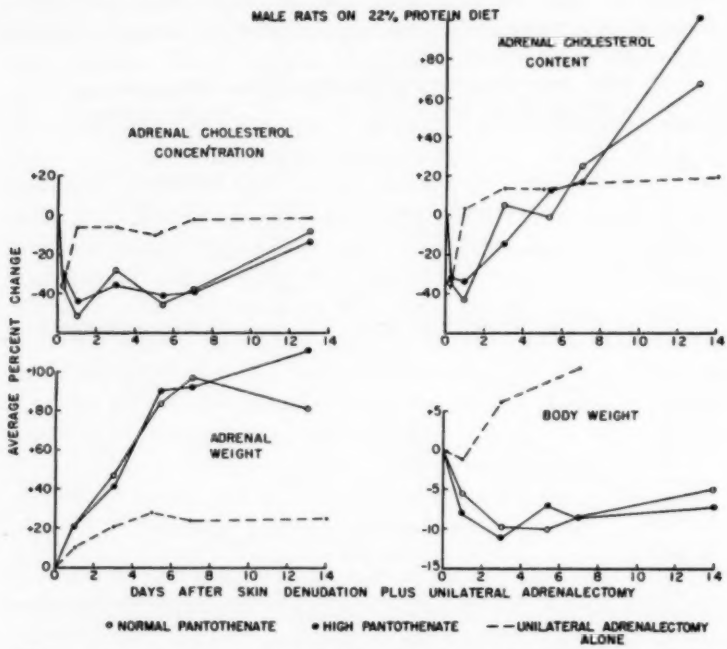

Fig. 4 The average per cent change in adrenal cholesterol concentration, adrenal cholesterol content, adrenal weight and body weight is shown for male rats on a 22% protein diet containing either normal (o–o) or high pantothenate (•–•), from 6 hours to 14 days after skin denudation plus unilateral adrenalectomy. The change in the same values after unilateral adrenalectomy alone is shown for male rats on a 22% protein normal pantothenate diet (– – –).

bined surgical procedure for 7 days and about 60% for 14 days. Data are presented only on rats that were in good condition at the time of sacrifice. The response in body weight was similar in both diet groups. The weight of the rats decreased for the first three days after the combined stress, and after 5 days tended to increase. The initial increase

in adrenal weight was similar in both broups. By 14 days the adrenals of the rats on the high pantothenate intake were heavier than in the animals on the normal pantothenate intake. Adrenal cholesterol concentration decreased more than 40% at 24 hours after the combined stress in both groups of animals. It remained at about this level for 7 days and was not influenced by the pantothenate content of the diet. The total cholesterol of the adrenals also decreased for the first 24 hours but thereafter increased steadily due to concomitant increase in adrenal weight.

The figure shows that the more severe stress of skin denudation plus unilateral adrenalectomy was associated with a greater and more prolonged decrease in adrenal cholesterol concentration, a greater increase in adrenal weight, a slower initial rise in adrenal cholesterol content, and with a decrease rather than an increase in body weight. The pantothenate level of the diet did not seem to influence these changes.

DISCUSSION

Unilateral adrenalectomy provides a reasonably standard form of acute stress. The rate of survival is good and, except for a brief interruption in weight gain, the operation is not associated with any general metabolic disturbances. Its primary advantage for studies of this kind is that it is possible to compare each animal with itself as a control.

In our experiments, pantothenic acid, ascorbic acid and protein all appear to have influenced the adrenal response of the rat following unilateral adrenalectomy. The rats receiving large supplements of pantothenic acid showed a greater increase in adrenal weight than did the normal controls during the second week after the injury. Supplementation with pantothenate did not influence the changes occuring in adrenal cholesterol concentration after stress. In animals on the high-ascorbic acid diet the restoration of adrenal cholesterol concentration was delayed after stress, and during the second week the increase in adrenal weight was greater than in the control animals. In rats on the low-protein diet

the adrenal cholesterol concentration 24 hours after the stress was lower than in rats on a normal protein diet.

The fact that pantothenic acid and ascorbic acid both were associated with a greater increase in adrenal weight the second week after stress suggests that there may be some common factor in the effect of these two fractions on the responses of the adrenals to stress. Adrenal hypertrophy following stress has been attributed to an increased output of the adrenocorticotropic hormone (Selye, '50). More recently, it has been suggested that a specific adrenal weight-maintaining factor may be secreted by the anterior pituitary (Mason, '53). The fact that the adrenals of animals on a high-pantothenate or high-ascorbic acid diet increased more in weight by 14 days after unilateral adrenalectomy than did the adrenals of control animals, suggests the possibility either of a greater output of an adrenal weight maintaining hormone under these circumstances or an increased capacity of the adrenal to respond to stimulus from the pituitary. The latter possibility appears more likely in view of the fact that ascorbic acid has been reported to enhance the effect of ACTH on adrenal weight in hypophysectomized rats (Dugal and Therien, '52). On the other hand, it has been reported that a high intake of ascorbic acid increased resistance to cold and inhibited adrenal hypertrophy in normal rats during cold stress (Dugal and Therien, '49; Dugal and Fontier, '52).

In earlier experiments from this laboratory, a deficiency in pantothenate was found to be associated with a delay in the restoration of adrenal cholesterol after stress (Dumm et al., '53). This was attributed to a deficiency in coenzyme A, an essential factor in cholesterol synthesis. There is no evidence in the present study to suggest that an excess of pantothenate influenced cholesterol synthesis in any way. Excess ascorbic acid, on the other hand, delayed the restoration of adrenal cholesterol concentration after surgical stress. Other observers have reported that a high intake of ascorbic acid was associated with an increase in adrenal cholesterol

after cold stress (Eisenstein and Boniface, '52). The difference between the latter results and ours may be related to the different forms of stress employed.

Adrenal changes in animals on protein-deficient diets have been attributed to diminished ACTH production(Selye, '46; Gershberg and Ralli, '52). It may be that the higher initial level of adrenal cholesterol in rats on a 16% protein diet and its slower return to normal following stress reflect a diminished output of ACTH compared to that of rats on a 22% protein diet. The prolonged delay in the resumption of growth by the rats on the 16% protein diet following unilateral adrenalectomy is also evidence of the inadequacy of this diet for animals subjected to surgical stress.

A comparison of the adrenal changes following the combined operations of unilateral adrenalectomy and skin denudation with those observed in rats on similar diets following unilateral adrenalectomy alone (figure 4) demonstrates the difference in the adrenal responses to a severe compared to a moderate stress. Both the prolonged depression in adrenal cholesterol concentration and the marked increase in adrenal weight which occurred after severe stress were probably associated with an increased production of ACTH and have also been observed after other forms of severe stress (Selye, '50). The severity of the stress was clearly an important factor in determining the magnitude and duration of the adrenal response both as to weight and cholesterol concentration.

The sex of the animals also influenced the time required to restore the pre-stress levels of adrenal cholesterol. Observations on the adrenal responses of male rats cannot be directly applied to female rats.

The experiments do not provide any indication of the absolute magnitude of the output of adrenal steroids by rats in various nutritional conditions when subjected to surgical stress. Rather they measure the time required for the rats to increase the synthesis of adrenal cholesterol in order to meet the greater demand for adrenal steroids that

follows stress. Nutritional factors which have been reported to modify the overall response of animals to stress also influenced the adrenal response, either in respect to adrenal cholesterol, adrenal weight or both.

SUMMARY

Adrenal weight and cholesterol have been measured in rats on various diets after unilateral adrenalectomy and after the combined operations of unilateral adrenalectomy plus skin denudation. In rats on a high-pantothenate diet, the increase in adrenal weight was greater than in rats on a normal-pantothenate diet during the second week after either unilateral adrenalectomy alone or after unilateral adrenalectomy plus skin denudation. A high intake of ascorbic acid was also associated with a greater increase in adrenal weight the second week after unilateral adrenalectomy. The restoration of adrenal cholesterol was slower in rats on a high intake of ascorbic acid than in rats on a normal diet, but was not affected by a high pantothenate intake. In rats on a 16% protein diet, adrenal cholesterol concentration was lower 24 hours after unilateral adrenalectomy than it was in rats on a 22% protein diet. The restoration of adrenal cholesterol occurred more slowly in female than in male rats on a normal-pantothenate 22%-protein diet. The more severe stress (unilateral adrenalectomy plus skin denudation) caused a greater increase in adrenal weight and a more prolonged depression of adrenal cholesterol concentration than did the moderate stress (unilateral adrenalectomy).

ACKNOWLEDGMENT

We are indebted to Miss Elisabeth Street for the statistical analyses of these data.

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NITROGEN UTILIZATION DURING CALORIC RESTRICTION

I. THE EFFECT OF DIETARY FAT CONTENT [1]

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TWO FIGURES

(Received for publication February 14, 1955)

In military operations individuals are sometimes cut off from food supply channels and must exist on limited amounts of food and water. To provide guidance for the design of a suitable survival food packet for use under such circumstances by the Armed Forces, systematic studies of nitrogen metabolism at restricted caloric intakes have been conducted. Since the nitrogenous end products produced when protein is catabolized for energy require additional urinary water for excretion, knowledge of the extent of protein utilization when fed at low calorie levels is important for prevention of dehydration when the intake of water is also limited. Special emphasis has, therefore, been placed on nitrogen utilization to ascertain the feasibility of incorporating in the survival food packet a suitable quantity of protein to spare and replenish body tissue protein.

Reports pertaining to the influence of the source of nonprotein calories on nitrogen metabolism have been contradictory. Maignon ('33) in a review of the literature pointed out

[1] "This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned no. 528 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense."

that many early workers, notably Voit and Atwater, reported improved protein utilization when protein-carbohydrate mixtures were fed as opposed to protein-fat combinations. His own experimental work, however, indicated that in diets of moderate egg albumin content, carbohydrate and fat exerted similar effects but that fat was superior when the protein concentration was increased. This led him to suggest that fat has a specific metabolic role in protein metabolism apart from the energy supplied. In a series of experiments with growing and mature rats, Forbes and coworkers ('46a, b, c) concluded that variation in fat from 2 to 30% did not affect nitrogen retention of diets of approximately 20% protein content. French et al. ('48) found no effect in similar 7% protein diets. Rosenthal ('52) observed that the nitrogen balance index of casein was unaffected by variation in dietary fat from 5 to 85% of the calories when the full caloric requirement was supplied to the dog; at energy intakes 25% of normal a high fat diet reduced the index, but this damaging effect could be offset by doubling the protein content of the diet.

Nitrogen excretion was found to be much greater in protein-depleted rats fed essentially fat-free or low-fat diets than in those given diets containing 15 or 20% of fat when the food intake was limited to 25% of normal; no differences were found at food intakes above 50% of normal (Willman et al., '47). Fox and Swanson ('54) reported that this increased nitrogen excretion was associated with a shortened survival time and further that the sparing action of fat was not related to essential fatty acids or to the degree of saturation of the fat. In protein-depleted rats subjected to hepatectomy, a 30% fat diet was superior to a pair-fed isonitrogenous 3% fat diet in the promotion of nitrogen balance and regeneration of liver protein (Rogers et al., '51).

In a comprehensive review, Munro ('51) concluded that in the normal animal both carbohydrate and fat promote protein utilization, but that carbohydrate exerts greater sparing action on endogenous nitrogen metabolism than does fat.

The divergence of opinion reported may be attributed to: (1) the complete absence of fat or of carbohydrate, (2) the source of or limitation in available protein, or (3) the nutritive state of the animal. It was therefore deemed advisable to study the total nitrogen balance of well-nourished animals fed diets low or high in fat at three levels of caloric intake and with two protein sources.

PROCEDURE

Male albino rats, Sprague-Dawley strain, were fed for two weeks on a commercial stock diet with the intake controlled to a level which maintained body weight constant at 300 gm. The maintenance energy requirement under the conditions of this study was found to be 46 Cal. per day. The environmental temperature was 76 to 80°F., with 40% relative humidity; feeding was limited to 7 hours daily and most animals consumed the food in 4 hours.

At the end of this standardization period, 8 animals were sacrificed for initial data on carcass and liver composition and 13 experimental groups of 6 animals each were designated in accordance with the experimental plan shown in table 1. The diets supplied at each calorie level approximately 53 mg of nitrogen/100 gm rat body weight/day, the minimum requirement suggested by Goettsch ('51), and were supplemented with all the essential B vitamins.[2] Vitamins A, D and E were given by dropper twice weekly.[3] The diets included 4% of Salts IV (Phillips and Hart, '35) and sucrose to make 100%. Animals fed reduced caloric intakes consumed their daily allowance in less than one hour.

A 4-day restriction period was selected because the degree of body weight loss attained is comparable to that anticipated in humans under military survival conditions and because dif-

[2] Vitamins supplied in milligrams per 100 gm of diet: Thiamine hydrochloride 0.50; riboflavin 0.50; pyridoxine hydrochloride 0.25; nicotinamide 2.00; calcium pantothenate 2.00; inositol 10.00; para-amino benzoic acid 5.00; biotin 0.01; folic acid 0.10; choline chloride 100.00; vitamin K 0.10 and vitamin B_{12} 50μg.

[3] 3000 I.U. of vitamin A, 30 I.U. of vitamin D and 2.5 mg of alpha-tocopherol.

ferences between dietary managements tend to disappear after this time (Calloway and Spector, '53). Animals were weighed daily during the restriction phase and were sacrificed on the morning of the 5th day. Urine and feces were collected by the method of Wissler et al. ('48) on boric acid-impregnated filter paper. Two urine collections were made on each rat representing two-day periods; feces were pooled for the entire 4-day period. The contents of the gastrointes-

TABLE 1

Experimental plan

CALORIC INTAKE	FAT [1]	PROTEIN		NITROGEN INTAKE
% of requirement	*% of diet*	*Source*	*% of diet*	*mg/day*
100	30	Egg albumin	14.00	161
50	30	Egg albumin	28.00	162
25	30	Egg albumin	56.00	164
100	5	Egg albumin	10.75	157
50	5	Egg albumin	21.50	153
25	5	Egg albumin	43.00	161
100	30	Milk [2]	14.20	159
50	30	Milk	28.40	165
25	30	Milk	56.80	164
100	5	Milk	10.90	157
50	5	Milk	21.80	155
25	5	Milk	43.60	164
0	..	...	....	0

[1] Hydrogenated coconut oil (Durkee's 2x oil).

[2] Mixture of casein and lactalbumin as derived from whole milk (Cas-al, Crest Foods Co.).

tinal tract were added to the fecal collection at sacrifice and the excreta analyzed for total nitrogen content by the macro-Kjeldahl method (A.O.A.C., '50). The livers were removed and weighed immediately and the carcasses weighed, sealed in polyethylene bags and frozen. The entire carcass was then ground three times in an electric grinder with dry ice added to prevent thawing. Livers and carcasses were analyzed for nitrogen and fat (A.O.A.C., '50).

The data were analyzed statistically by analysis of variance (Kempthorne, '52). Where values are stated as different

they have been proved statistically so at or below a 5% level of significance.

RESULTS

Body weight change

Body weight losses varied inversely with the caloric intake, but there was no significant effect of the level of fat in the diet or of source of protein (fig. 1). The mean total loss of

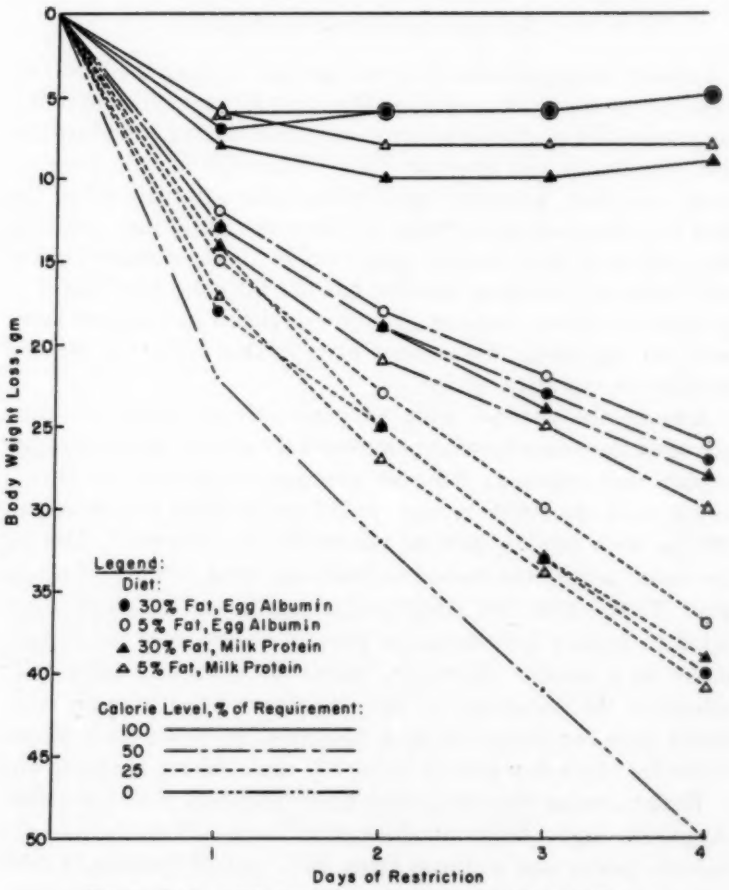

Fig. 1 Body weight loss at varying levels of caloric intake.

2% for all full-fed groups was confined to the first day, body weight remaining essentially constant thereafter. Body weight loss of this magnitude has been found consistently after isocaloric substitution of a purified diet for a commercial mixed feed. Average weight losses of all the restricted groups were: 9% at half the caloric requirement, 13% at one-quarter the caloric requirement and 17% in fasting.

Nitrogen excretion and balance

Urinary nitrogen excretion was greater during the first two days of restriction than during the second (table 2), due to the carry-over from the high nitrogen intake during standardization (Calloway and Spector, '53). When the caloric requirement was met, nitrogen equilibrium was attained after the first two days of adjustment to decrease in dietary protein. Egg albumin was slightly superior to milk proteins in the promotion of nitrogen balance, but the fat content of the diet exerted no effect. Among groups restricted in calories there were no significant differences attributable either to type of protein or quantity of fat.

Among the groups with varying caloric intake urinary nitrogen excretion became progressively greater as the energy supply was reduced; from an average of 132 mg on days 3 and 4 in all the 100% groups, to 192 mg in those fed 50%, and 268 mg with intakes 25% of the caloric requirement. During the same period the fasted animals excreted 196 mg of nitrogen. There were no statistically significant differences related to dietary fat content or protein source over the experiment as a whole. However, when the diet was calorically adequate the excretions of the egg albumin-fed animals were lower than for those fed milk proteins, the mean daily excretions for the 4-day period being 137 and 163 mg respectively.

Both proteins employed were approximately 97% digestible. Apparent digestibility or absorption increased slightly as the caloric intake was reduced from 96% in full feeding to 98% at one-quarter of this.

The only variable with marked influence on nitrogen balance was caloric intake. In figure 2, this direct correlation between negativity of nitrogen balance and caloric restriction is shown. The mean daily negative balance was 6 mg of nitrogen at 100% intake, increasing progressively to 63 mg at 50%, 128 mg at 25% and 208 mg in fasting.

TABLE 2

Nitrogen excretion and balance at varying levels of caloric intake

REGIMEN	URINARY N EXCRETION		FECAL N	N BALANCE	PROTEIN DIGESTIBILITY
	Days 1–2	Days 3–4	Days 1–4	Days 1–4	
	mg/day	*mg/day*	*mg/day*	*mg/day*	%
100% CR[1]					
Egg, 30% fat	169	118	14	+ 4 ± 4[2]	96.3
Egg, 5% fat	150	111	15	+ 11 ± 4	95.5
Milk, 30% fat	181	146	13	— 18 ± 5	96.8
Milk, 5% fat	170	153	16	— 21 ± 4	95.1
50% CR					
Egg, 30% fat	230	204	11	— 66 ± 7	98.1
Egg, 5% fat	214	178	14	— 57 ± 8	96.1
Milk, 30% fat	241	185	13	— 61 ± 4	97.1
Milk, 5% fat	219	202	13	— 69 ± 2	96.8
25% CR					
Egg, 30% fat	285	264	11	— 122 ± 18	98.5
Egg, 5% fat	286	261	12	— 124 ± 9	98.0
Milk, 30% fat	302	269	12	— 133 ± 8	97.9
Milk, 5% fat	294	278	12	— 134 ± 9	97.8
Fasted	204	196	8	— 208 ± 13	
Summated means:					
Egg	222	190	13	— 59	97.1
Milk	234	206	13	— 73	96.9
30% Fat	235	198	12	— 66	97.4
5% Fat	223	197	14	— 66	96.5
100% CR	168	132	14	— 6	95.9
50% CR	226	192	13	— 63	97.0
25% CR	292	268	12	— 128	98.0

[1] CR = Calorie requirement.

[2] Standard error.

Tissue composition

The percentage of liver nitrogen increased progressively with decrease in caloric intake, from 11.48 to 12.78% (table 3). This apparent increase was largely a function of diminished concentration of liver fat from 9.11 to 5.93%. Where the percentage nitrogen content was lower than the control value, among the full-fed groups, the change was due to the dilution effect of increased fat content. All of the values reported are within the normal range.

The total nitrogen content of the livers of all experimental groups was reduced from control values, as would be antici-

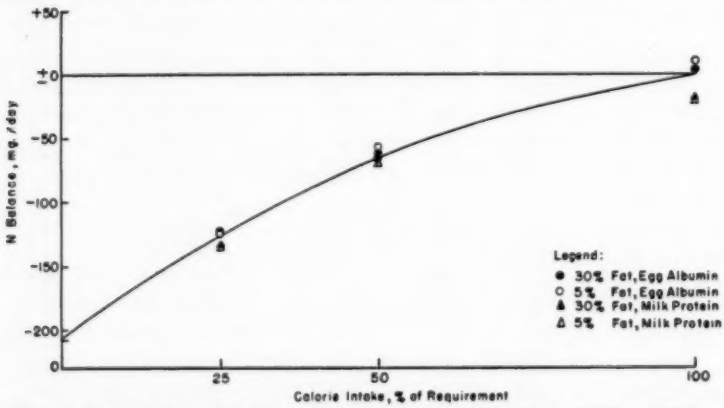

Fig. 2 Relationship between nitrogen balance and caloric intake during a 4-day period.

pated in response to diminished nitrogen or caloric intake. These losses were least in the groups fed the full energy requirement and of these groups the egg-fed animals exhibited smaller losses than did those given milk proteins, 7% as compared with 14%. At 50 and 25% caloric intakes liver nitrogen losses were similar (the means being 17 and 18%), variation among and within groups was large, and there was no consistent relationship to either dietary protein or fat. All liver nitrogen reserves must not have been exhausted in these restricted groups since the fasted animals expended almost double the amount of liver nitrogen, 32%.

Total liver fat content varied directly with the level of caloric intake, dropping from a mean of 327 mg at 100% of the caloric requirement to 143 mg in fasting. Measured over the experiment as a whole, retention or deposition of liver fat appeared to be favored by feeding egg albumin or 30% fat or both. The maximum effect was found in the 100%-egg

TABLE 3

Liver composition of animals fed varying levels of caloric intake for 4 days

REGIMEN	NITROGEN		FAT	
	(dry basis)	Total	(dry basis)	Total
	%	mg	%	mg
Control	11.98	453 ± 13 [1]	8.42	318 ± 15
100% CR [2]				
Egg, 30% fat	10.75	421 ± 19	11.15	437 ± 23
Egg, 5% fat	11.62	423 ± 11	8.64	314 ± 6
Milk, 30% fat	11.35	390 ± 10	8.69	299 ± 19
Milk, 5% fat	12.22	396 ± 11	7.97	258 ± 11
50% CR				
Egg, 30% fat	12.12	329 ± 19	8.64	233 ± 9
Egg, 5% fat	12.48	376 ± 11	7.40	223 ± 9
Milk, 30% fat	12.11	369 ± 21	7.17	219 ± 15
Milk, 5% fat	12.58	405 ± 13	5.86	194 ± 4
25% CR				
Egg, 30% fat	12.88	392 ± 7	6.61	202 ± 6
Egg, 5% fat	12.74	372 ± 13	6.26	182 ± 4
Milk, 30% fat	12.84	373 ± 18	5.72	166 ± 11
Milk, 5% fat	13.06	365 ± 15	6.31	176 ± 7
Fasted	12.78	310 ± 14	5.93	143 ± 5
Summated means:				
Egg	12.10	386	8.12	265
Milk	12.36	383	6.95	219
30% fat	12.01	379	8.00	259
5% fat	12.45	390	7.07	225
100% CR	11.48	408	9.11	327
50% CR	12.32	370	7.27	217
25% CR	12.68	376	6.22	182

[1] Standard error.

[2] CR = Calorie requirement.

albumin-30% fat group where the quantity of liver fat increased 37% over the controls. Differences due to dietary protein or fat disappeared as the caloric intake was reduced.

Changes in carcass composition paralleled those in liver composition but were of smaller magnitude (table 4). A decrease in carcass percentage nitrogen was concomitant with an increase in percentage of fat in the groups given full

TABLE 4

Carcass composition of animals fed varying levels of caloric intake for 4 days

REGIMEN	NITROGEN		FAT	
	(wet basis)	Total	(wet basis)	Total
	%	gm	%	gm
Control	3.62	10.08	6.96	19.34
100% CR[1]				
Egg, 30% fat	3.49	9.68	9.20	25.50
Egg, 5% fat	3.51	9.72	8.14	22.55
Milk, 30% fat	3.59	9.78	8.23	22.38
Milk, 5% fat	3.57	9.80	8.29	22.67
50% CR				
Egg, 30% fat	3.76	9.64	5.96	15.26
Egg, 5% fat	3.74	9.70	7.04	18.25
Milk, 30% fat	3.76	9.56	6.45	16.38
Milk, 5% fat	3.77	9.47	5.78	14.51
25% CR				
Egg, 30% fat	3.86	9.40	4.76	11.62
Egg, 5% fat	3.88	9.47	4.54	11.10
Milk, 30% fat	3.83	9.30	5.64	13.70
Milk, 5% fat	4.02	9.58	3.21	7.66
Fasted	3.93	9.04	3.18	7.40
Summated means:				
Egg	3.71	9.60	6.61	17.38
Milk	3.76	9.58	6.27	16.22
30% Fat	3.72	9.56	6.71	17.47
5% Fat	3.75	9.62	6.17	16.12
100% CR	3.54	9.74	8.46	23.28
50% CR	3.76	9.59	6.31	16.10
25% CR	3.90	9.44	4.54	11.02

[1] CR = Calorie requirement.

feeding as compared with controls. In restricted groups the nitrogen concentration per gram of tissue rose as fat diminished. As in the livers, all groups lost some nitrogen but these losses were minor: 3% with complete caloric intake, 4 to 8% in restricted feeding and 10% in fasting. The type of protein or level of fat employed exerted no consistently significant effect.

Total fat content varied directly with the energy supplied, from a mean of 23.28 gm in the 100% caloric requirement groups which represented a 20% increase over the control value to 7.40 gm in fasted animals, a 62% loss.

SUMMARY AND CONCLUSIONS

Well-nourished mature rats were fed for 4 days diets composed of egg albumin or milk proteins containing either 30 or 5% of fat at three levels of caloric intake: 100, 50 or 25% of the determined requirement. Fasted and normal composition controls were employed. The results of this study are summarized as follows:

1. Body weight loss was inversely proportional to the level of calories fed and was unaffected by the composition of the diets.

2. All calorie-restricted groups were in negative nitrogen balance, the degree of negativity being directly related to the caloric intake. No superiority of level of fat or of type of protein was evident.

3. The same qualitative changes in composition were found in both liver and carcass, but these changes were magnified in the liver. With respect to nitrogen content, losses were least in groups fed a full diet, were greater but approximately equal in groups receiving 50 and 25% of the caloric requirement, and greatest in the fasted group. The level of fat was directly proportional to the caloric intake. There was no consistent relationship between tissue losses and dietary fat content or protein source.

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NITROGEN UTILIZATION DURING CALORIC RESTRICTION

II. THE EFFECT OF VARIATION IN NITROGEN INTAKE [1]

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ONE FIGURE

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Numerous investigations have shown that nitrogen utilization is a function of both caloric and protein intake. Benditt et al. ('48) found increased nitrogen utilization by the protein-depleted rat up to intakes of 6 gm of protein per kilogram per day and up to 1240 Cal./m^2/day when the factors were varied independently. However, it was demonstrated by Allison and co-workers ('46) that the nitrogen balance index in the normal dog was unaltered provided that 50% or more of the caloric requirement was met; the index was markedly reduced at the 25% level of energy intake. In studies employing protein-depleted rats, supplementation with egg protein or methionine was effective in sparing body protein only when 50% or more of the energy requirement was supplied (Stevenson et al., '46). Rosenthal ('52) reported a normal index with a low fat, low-casein (0.15 gm N/kg) diet fed at 25% caloric requirement, and positive nitrogen balance with a reduction in index when the casein nitrogen was in-

[1] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned no. 529 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

creased to 0.30 gm/kg. Because of the techniques involved in these studies all of the animals employed were restricted in protein for variable lengths of time.

In the experiments presented here the relationship between nitrogen intake and utilization was examined in normal adult rats restricted to half the caloric requirement.

PROCEDURE

Male albino rats, Sprague-Dawley strain, were standardized for two weeks as described in the preceding paper (Calloway and Spector, '55). Three series were conducted: in series I, commercial stock diet was fed during the standardization period; in series II, a complete 18% casein diet; and in series III, commercial stock diet for two days (until the shipping weight loss was recovered) and an 18% casein diet for the remaining 12 days. In each series body weight was maintained at 300 gm.

After standardization, 5 or 6 control animals from each series were sacrificed for tissue analysis. During the next 4-day period an egg albumin diet was fed to supply half the previous caloric intake and graded levels of nitrogen as shown in table 1. The nitrogen content of the diet was increased at the expense of sucrose; fat, salts and vitamins [2] were held constant. In series I and II the diets contained 30% fat by weight and in series III, 5%. The amounts of nitrogen fed in series I and II were at or below the normal requirement, 160 mg per day; in series III the nitrogen consumptions were increased from 160 to 600 mg. In addition, fasted and protein-free fed controls were employed. Six animals comprised each group in series I and III; 5 animals per group were used in series II.

[2] Vitamins were given by dropper daily to supply the following amounts in micrograms:

Thiamine 25; riboflavin 50; pyridoxine 25; nicotinamide 200; calcium pantothenate 200; para-amino benzoic acid 500; biotin 1; folic acid 5; vitamin B_{12} 1; vitamin K 10; and inositol 1 mg; choline chloride 10 mg; α-tocopherol 1 mg; vitamin A 1,200 I.U.; vitamin D 20 I.U.

TABLE 1

Diet plan

	SERIES I	SERIES II	SERIES III
Standardization diet	Commercial stock [1]	18% casein	Commercial stock, 2 days 18% casein, 12 days
Calorie intake, Cal./day	46	47	52
Nitrogen intake, mg/day	460	270	315
Restriction			
Egg albumin, % of diet	0–28.2	0–28.2	0–90
Fat,[2] % of diet	30	30	5
Caloric intake, Cal./day	23	23	25
Nitrogen intake, mg/day			
a	0	0	0
b	69	81	162
c	98	119	347
d	126	160	505
e	156		604

[1] Purina Laboratory Chow.

[2] Hydrogenated vegetable fat.

TABLE 2

Body weight loss after 4 days of caloric restriction

SERIES	NITROGEN INTAKE	WEIGHT LOSS
	mg/day	%
	At 50% caloric requirement	
I	0	11.8 ± 0.3 [1]
	69	11.2 ± 0.4
	98	11.1 ± 0.8
	126	10.6 ± 0.6
	156	10.5 ± 0.5
II	0	8.0 ± 0.2
	81	7.3 ± 0.3
	119	8.0 ± 0.4
	160	6.9 ± 0.6
III	0	7.2 ± 0.4
	162	6.3 ± 0.2
	347	7.5 ± 0.4
	505	7.4 ± 0.2
	604	5.6 ± 0.2
	Fasting	
I		17.0 ± 0.2
II		14.4 ± 0.3
III		15.1 ± 0.6

[1] Standard error.

Urine and feces were collected and analyzed in the manner described in the preceding paper (Calloway and Spector, '55). Livers were analyzed as before and total serum protein as determined by the copper sulfate specific gravity method (Phillips et al., '50) was measured in series II.

RESULTS

Body weight changes

Body weight losses were independent of the level of nitrogen fed during caloric restriction. Average losses of the groups were 10.5 to 11.8% in series I, 6.9 to 8.0% in series II, and 5.6 to 7.2% in series III (table 2). Losses in fasted animals were greater. These variations in weight loss among series are attributable to the diet fed during standardization (Calloway and Spector, '53).

Nitrogen utilization

The degree of negative nitrogen balance was inversely proportional to the nitrogen intake (fig. 1, table 3). Positive nitrogen balance was not attained although it was approached in the group fed 604 mg of nitrogen daily, approximately 2 gm per kilogram of body weight. The slope of the line in figure 1 indicates that positive balance might be reached at higher intakes.[3] A difference in response among the three series is evident, the series II groups being in less negative balance than those of series I. At the 160 mg intake, the balances were significantly different ($p < .001$) although the balances were essentially the same when no nitrogen was fed.

The percentage of nitrogen utilization, shown in table 3, was calculated by the formula:

$$\text{Per cent utilization} = \frac{\text{Absorbed N} - (\text{Urinary N} - \text{Urinary N on protein-free regime})}{\text{Absorbed N}} \times 100.$$

This formula is the same as that derived by Allison

[3] An attempt was made to feed pure egg albumin but the data are omitted because the animals had to be force-fed and diarrhea was marked. However, under these conditions positive balance was obtained at an intake of 750 mg of nitrogen per day.

and Anderson ('45) for obtaining nitrogen balance index but in this case expresses nitrogen utilization as percentage of that absorbed; since the term "nitrogen balance index" has a specific derivation its use has been avoided here. The application of the formula in this instance differs from that of the originators in that a group of animals fed a protein-

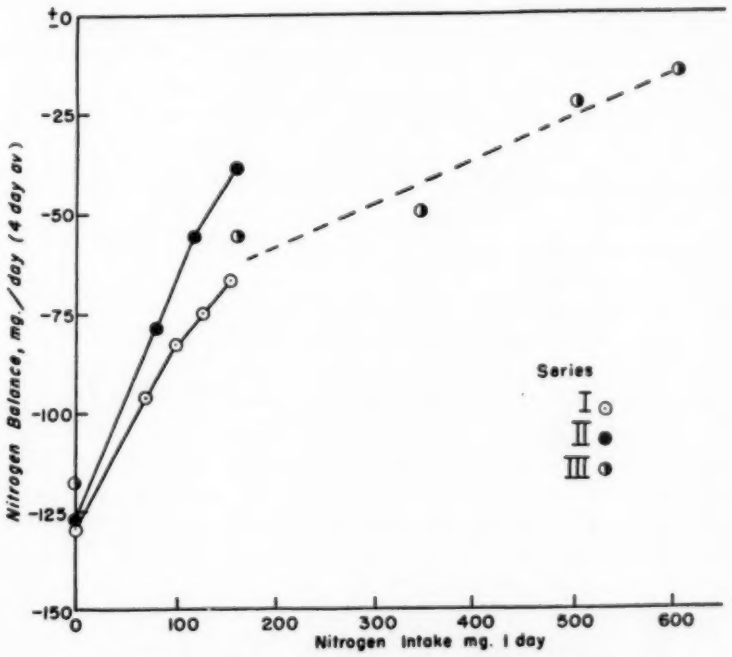

Fig. 1 Nitrogen balance during 4 days of 50% calorie restriction.

free diet provided the base line rather than each animal serving as its own control. This technique was selected in order to obviate the possible complication of even a short period of protein restriction. In each series percentage utilization diminished as the dietary level of nitrogen increased, representing progressively larger fractions of dietary protein expended for energy. In no case, however, was there

TABLE 3

Nitrogen utilization during 4 days of caloric restriction

SERIES	NITROGEN INTAKE	ABSORBED NITROGEN [1]	URINARY NITROGEN	NITROGEN BALANCE	NITROGEN UTILIZATION [2]	REDUCTION OF NITROGEN DEFICIT COMPARED WITH	
						Fasting	Equal non-protein calories
	mg/day	*mg/4 days*	*mg/4 days*	*mg/4 days*	%	%	%
				At 50% caloric requirement			
I	0	0	488	— 518 ± 8 [3]		38	
	69	269	617	— 386 ± 32	52		25
	98	387	681	— 333 ± 21	50		36
	126	495	762	— 302 ± 17	45		42
	156	617	849	— 269 ± 29	41		48
II	0	0	469	— 508 ± 10		32	
	81	316	593	— 315 ± 15	61		38
	119	471	653	— 222 ± 29	61		56
	160	628	749	— 160 ± 29	55		68
III	0	0	432	— 470 ± 29		39	
	162	642	829	— 225 ± 26	38		52
	347	1374	1536	— 200 ± 19	20		57
	505	1982	2041	— 90 ± 24	19		81
	604	2383	2415	— 60 ± 38	17		87
				Fasting			
I			801	— 834 ± 36			
II			714	— 744 ± 29			
III			741	— 772 ± 25			

[1] Absorbed nitrogen = nitrogen intake — (fecal nitrogen — fecal nitrogen, protein-free).

[2] Nitrogen utilization, $\% = \frac{AN - (UN\text{-}UN \text{ pro-free})}{AN} \times 100$.

[3] Standard error.

quantitative excretion of dietary protein, i.e., zero utilization. In the groups of series II, utilization was superior to that obtained in I and III; when the intake was 160 mg of nitrogen daily the values were 55% as compared with 41 and 38%.

Analysis of these data in terms of what they represent in body economy shows that feeding half the caloric requirement reduced body losses by 32 to 39% as compared with fasted animals. When the normal nitrogen requirement was met in addition, further savings of 48 to 68% were induced. At the highest level shown, 604 mg daily nitrogen intake, the reduction of nitrogen deficient was 87% above that due to calories alone. In order to increase the net protein saving from 52 to 87%, however, the intake had to be raised 377% and the concomitant increase in urinary nitrogen excretion was 291%.

The nitrogen retention reflected in the percentage utilization recorded here is markedly less than that reflected in the nitrogen balance indexes of egg albumin as reported: 0.91 in man, 1.14 in the dog and 0.94 in the rat under conditions of full feeding (Rutgers, '46–'50). The reason for the divergence of these data is not clear. Allison ('51) has stated that ". . . indexes are essentially constant and independent of the magnitude of the protein stores . . ." and are unaffected by 50% caloric deficit. Other factors to be considered as sources of variance are: (1) the variation in the base line of protein-free feeding and (2) the preceding diet of the animals. These factors will be elaborated in a subsequent paper.

Tissue composition

From table 4 it may be observed that there were distinct differences among the livers of control groups of the three series. These differences were based largely upon the size of the organs, since there was no significant difference in percentage content of moisture or of nitrogen. In the first series the livers weighed an average of 10.3 gm, 3.42 gm per

[1] Standard error.

100 gm of body weight; in series II, these values were 12.0 and 4.03 gm; and in series III, 14.0 and 4.62 gm. Because of the similarity in percentage nitrogen content, this variation in organ size resulted in significantly different total amounts of liver nitrogen ($p < 0.05$).

TABLE 4

Liver composition of rats fed varying amounts of nitrogen and calories

SERIES	NITROGEN INTAKE	LIVER WET WEIGHT	MOISTURE	NITROGEN	
				(dry basis)	Total
	mg/day	*gm*	%	%	*mg*
			Control		
I	460	10.3	70.2	12.0	369 ± 8[1]
II	270	12.0	72.3	12.2	404 ± 14
III	315	14.0	72.3	12.8	496 ± 17
			After 4 days of 50% caloric restriction		
I	0	10.2	72.4	12.6	354 ± 9
	69	10.6	72.6	12.5	364 ± 8
	98	10.3	72.3	12.5	358 ± 10
	126	10.3	72.3	12.6	356 ± 14
	156	9.8	72.5	12.8	350 ± 10
II	0	8.5	68.8	11.5	304 ± 7
	81	8.9	67.6	11.1	318 ± 4
	119	9.2	67.0	11.1	337 ± 15
	160	9.7	67.7	11.0	346 ± 12
III	0	9.9	72.8	12.4	335 ± 17
	162	10.6	72.6	12.4	362 ± 10
	347	10.5	71.9	12.2	360 ± 17
	505	11.4	72.4	12.2	386 ± 7
	604	11.3	72.0	12.1	383 ± 12
			After 4 days of fasting		
II		7.4	67.0	11.5	280 ± 15
III		8.9	72.3	12.7	312 ± 6

[1] Standard error.

It is readily apparent that after restriction there were no significant changes from control liver nitrogen in the first series where that control value was low; in the second and third series where measurable losses were encountered, those

losses tended to diminish with increasing nitrogen intake, but the response was not uniform. The process of restriction served to eliminate the aberrant character of the series; that is, among the groups given 160 mg of nitrogen there were no significant differences and among the protein-free groups only the extremes (I vs. II, $p = 0.003$) were different. There was no correlation between nitrogen utilization as measured by nitrogen balance and by the changes in liver nitrogen content.

Neither kidney weight nor total serum protein showed significant changes as a result of either caloric restriction or alteration in dietary nitrogen. Kidney weight averaged 2.1 gm in controls and 2.0 gm after fasting; serum protein was 7.0 gm % initially and 6.9 gm % after fasting.

SUMMARY

Diets supplying graded amounts of egg albumin nitrogen, zero to 604 mg per day, were fed to normal adult rats limited to 50% of caloric requirement for 4 days. Nitrogen utilization was measured by nitrogen balance and by alteration in liver nitrogen content.

The degree of negative nitrogen balance was inversely proportional to nitrogen intake but positive balance was not attained. Nitrogen utilization diminished as the level of dietary nitrogen increased from 52 to 61% at 70 to 80 mg daily to 17% at 604 mg, indicating increased use of dietary protein for energy. Supplying half the caloric requirement reduced the nitrogen deficit 32 to 39% as compared with fasting and the addition of dietary nitrogen produced up to 87% sparing beyond that of the calories alone.

There was no significant correlation between liver nitrogen loss and nitrogen balance.

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LYSINE AND METHIONINE SUPPLEMENTATION OF ALL-VEGETABLE DIETS FOR HUMAN ADULTS[1]

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Nutrition teaching emphasizes the importance of animal sources of protein in the diet. This is based upon the general classification of animal proteins as "high quality" and the vegetable proteins as "poor quality". Although most of the data on the nutritive value of proteins has been obtained with animal experiments, there is no doubt that the general classification is also valid for human beings. Mitchell ('48) has summarized the available data. Of the proteins tested upon human subjects the biological values range from 41 for white flour to 78 for whole egg protein. Studies of population groups such as those of Orr and Gilks ('31) and of McCarrison ('32), comparing meat eaters to cereal eaters, have shown the over-all superiority in health and development

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of those consuming the high animal-protein diets. Comparisons of the population of the United States and similar populations with those consuming primarily vegetarian diets demonstrate a similar effect. This evidence cannot be considered definitive with regard to protein, since the intakes of many other nutrients are related to protein and other health hazards are associated with the economic and social conditions in different parts of the world.

The studies of Block and Mitchell ('46–'47) demonstrated that with few exceptions the biological value of proteins is determined by the amount and ratio of the essential amino acids supplied. In a few proteins, such as those of some raw legumes, the rate of release of the amino acids during digestion may be a determining factor in the over-all nutritive value, but the general correlation between biological value and amino acid composition leaves little doubt that the value of a protein for an animal depends primarily upon its content of the essential amino acids. The practical value of much of the data obtained with individual proteins is not readily evident since diets containing a single protein source are not often encountered and the ability of different proteins to supplement each other is well known. Data upon the supplementary value of various vegetable proteins are not extensive although this would appear to be of primary importance in the evaluation of vegetarian diets. Such studies have been summarized by Mitchell ('48) and indicate that the proteins supplied by various vegetables and cereals must supplement each other, and that the improvement in the biological value of the whole diet by the addition of animal proteins may not be as great as is often assumed. The minimum protein need for a 70 kg man consuming only white flour as a source of protein was estimated to be 42.1 gm per day by Bricker et al. ('45). The requirement when an all-vegetable mixed diet was fed was estimated to be 32.4 gm by Hegsted et al. ('46). With diets which contained 47 and 33% of the protein from animal sources the minimum requirement was estimated to be 27.6 and 27.1 gm, respectively,

by Bricker et al. ('45) and Hegsted et al. ('46). Thus, the improvement upon the addition of meat and other animal sources of protein to the diet composed of vegetables was not very great. The relatively high biological value of the proteins from potato and soy bean, corn germ, etc., is recognized. (See Mitchell, '48.)

Numerous studies, particularly the compilations of Block and Mitchell ('46–'47), indicate that the amino acids most likely limiting in vegetable protein are lysine and the sulfur amino acids, methionine and cystine. In view of the recent evidence from Rose ('53) that the adult human utilizes cystine much more efficiently as a substitute for methionine than does the growing rat upon which most of our data has been obtained, it appears that lysine may be the amino acid most limiting in dietaries containing only small quantities of high quality proteins. The recent view of Flodin ('53) emphasized this possibility. Studies upon some of the cereal proteins suggest that valine and threonine may also deserve consideration (Sure, '52; Pecora and Hundley, '51). The studies reported in this paper were designed to determine the effect of lysine supplements on nitrogen balance in human subjects fed all-vegetable diets.

EXPERIMENTAL

Young women, student dietitians of the Escuela de Dietistas of the Hospital Obrero, Lima, Peru, were the subjects. During the course of each study, they lived and took all meals in the hospital, but continued with their usual class and laboratory work. Individual data on the subjects of the three experiments are given in table 1. Six persons participated in both experiments two and three. Hemoglobin and hematocrit blood values were determined at least twice in each study. These were always within the normal range, with the exception of subject 9, whose hemoglobin was only 6.4 gm%. No other abnormalities were observed.

Each of the three experiments consisted of three periods, in which the diets described in table 2 were fed. The first

period varied in duration from three to 7 days, and the subjects received the so-called "depletion" diet. This was an all-vegetable diet low in total protein and in the third experiment also low in calories. Only during experiment 3 were

TABLE 1

Ages, heights and weights of subjects for nitrogen balance studies

SUBJECT	AGE	HEIGHT	WEIGHTS		
			Initial	End of "Depletion"	Final
	yr.	*cm*	*kg*	*kg*	*kg*
Experiment 1 (Dec. 1953)					
1	19	163	64.5		64.8
2	24	160	61.1		61.6
3	20	150	48.6		48.3
4	20	158	47.3		48.5
5	19	155	52.3		53.1
Experiment 2 (May 1954)					
6	20	152	49.5	48.6	48.5
7	21	157	48.2	48.5	48.3
8	20	152	47.3	46.8	46.9
9	19	146	45.4	46.0	45.4
10	20	151	49.1	48.8	49.2
11	19	156	48.2	48.3	48.5
12	19	154	46.8	46.5	46.3
13	21	146	44.5	44.2	44.7
Experiment 3 (Aug. 1954)					
6	21	152	50.6	48.8	48.5
7	21	157	48.9	47.4	48.1
8	20	152	47.3	45.7	46.3
10	20	151	51.4	49.2	50.0
11	19	156	49.2	48.1	48.9
12	19	154	48.4	47.3	47.5
14	22	149	51.1	49.1	50.1
15	21	152	50.7	49.4	50.0

collections of urine and stools made during the depletion period. During the second and third periods of 5 days each, the "basal" diets (table 2) were given with supplements of lysine, methionine and glycine as described for the individual experiments. The nitrogen balance for each subject was determined during the final two periods.

TABLE 2

Composition of diets used in balance studies

	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	(1) Depletion	(2) Basal	(3) Depletion	(4) Basal	(5) Depletion	(6) Basal
			(All values in grams per day per person)			
Bread	90	210	30	90	...	90
Noodles	25	75	25	25	...	25
Rice	30	125	50	50	...	50
Orange juice	180	180	180	180	...	180
Lemon juice	20	20	20	20	20	10
Avocado	...	100	...	100	...	100
Other fruits (apple, banana, pear, papaya, or pineapple)	450	400	450	400	500	400
Potato or yuca	100	...	100	100	250	100
Carrots	50	50	50	50	50	50
Cauliflower	50	100	50	50	50	50
Cucumber	50	...	...	...	...	...
Onion	50	50	30	40	40	40
String beans	50	50	50	50	50	50
Tomatoes	200	100	200	100	200	100
Butter	30	30	30	35	...	45
Cooking oil	40	40	40	40	20	20
Sugar	50	45	55	55	35	60
Sugar candies	25	25	25	25	...	25
Marmalade or jelly	50	45	60	60	...	60
Bouillion cubes	2 cubes	...	...	...	...	...
Soft drink	1 bottle	1 bottle	...	...	...	...
Nescafe	3	3	3	3	3–5	5
Protein ($N \times 6.25$)[1]	25.4	48.0	17.0	24.1	10.6	24.7
% of N from wheat prod.	49.5	65.6	34.1	48.5	0	47.4
% of N from cer. prod.	57.5	83.0	54.0	62.6	0	61.2
Solids (dry weight)[1]	456	600	418	480	234	470
Calories (calc'd)[1]	2060	2715	1985	2245	1035	2155

[1] Values for "basal" diets are average values for the two periods in each experiment.

The compositions of the all-vegetable diets are shown in table 2. The bread, made in the hospital bakery, contained no milk and the noodles contained neither milk nor eggs. After removal of non-edible parts, the uncooked foods were weighed individually and issued to the subjects. Each then prepared her own meal, using individual hot plates and utensils. The cooking was under strict supervision to insure uniform preparation and that all food was either eaten or kept for analysis. A student of the school, who was not an experimental subject, prepared identical meals which were analyzed. Slight variations in the methods of preparing the menus were permitted, but the daily total food consumption during each period was essentially the same.

During the first experiment some subjects failed to eat all of the food issued, while others ate more bread and marmalade than is shown in table 2. All uneaten food was kept for analysis, and all extra food was weighed and equivalent quantities analyzed. In the second and third experiments the amount of food was reduced and was always consumed. All subjects received one tablet containing the equivalent of 4 gm of calcium gluconate and one multivitamin capsule daily.[2] Carmine was used to mark the stool collections.

Pooled aliquots of the homogenized daily diets for each period were analyzed for nitrogen and total solids and during the third experiment for fat and ash, using standard procedures. In the first experiment pooled aliquots of the daily urine and stool collections for each of the 5-day metabolic periods were analyzed for nitrogen. In the second and third experiments, stool samples were again pooled for each metabolic period, but urinary nitrogen and creatinine determinations were made daily. Creatinine was determined by the method of Clark and Thompson ('49).

The lysine content of the diets was determined microbiologically using *L. mesenteroides* P-60 and the basal medium

[2] The calcium tablets were a gift from La Casa Sandoz; the multivitamin capsules were provided by Berkemeyer and Co., S. A. Peruvian representatives of Eli Lilly and Co.

of Horn et al. ('50). An appropriate aliquot of the pooled diets was refluxed for 24 hours with approximately 6 volumes of 4 N HCl. In the second experiment the acid hydrolyzate thus obtained was also analyzed for leucine, phenylalanine and isoleucine with *L. mesenteroides* P-60, and for threonine and valine with *S. faecalis* 8043 using the medium of Horn et al. ('50) Methionine was determined with *L. arabinosis* 17-5 using the oxidized casein media of Riesen et al. ('46). Tryptophan was determined by the method of Woolley and Sebrell ('45) after enzymatic hydrolysis with *L. arabinosis* 17-5. In the third experiment "basal" diets were analyzed for cystine. Appropriate aliquots of the diets were incubated for 4 days at 37°C. with saliva to hydrolyze the carbohydrate. The insoluble residue separated by centrifugation and washed with hot water was refluxed for 6 hours with approximately 5 volumes of 2 N HCl. The filtered hydrolyzate was analyzed for cystine using *L. mesenteroides* P-60 and a medium containing oxidized peptone (Lyman et al., '46). The nitrogen content of the hydrolyzate was determined by nesslerization and the cystine content related to total nitrogen content.

RESULTS

Experiment 1

Five persons participated in this study. During the three-day "depletion" period the all-vegetable diet provided 4.06 gm of nitrogen and approximately 2100 Cal. per day per person (diet 1, table 2). The "basal" diet (diet 2, table 2) providing about 7.7 gm nitrogen and about 2800 Cal. per day per person was given on the 4th day, and the first 5-day metabolic period was begun on the 5th day. The diets of this metabolic period were repeated during the second metabolic period which began on the 10th day. The bread, which provided about 45% of the total nitrogen, was equally distributed in each of the three meals. Noodles were issued for one of the main meals (lunch or dinner), while rice was given for the other. During the 5 days of the second metabolic

period each subject received a daily supplement of 0.9 gm of lysine, equally distributed during the three meals and dissolved in 60 ml of orange juice.

The average daily nitrogen balance for each subject during each of the two metabolic periods is given in table 3. All subjects were in positive nitrogen balance during the first (nonsupplemented) metabolic period; the average was 1.62 gm of nitrogen per day. During the second metabolic period

TABLE 3

Nitrogen balance with and without lysine supplementation

(Experiment 1)[1]

	SUBJECTS				
	1	2	3	4	5
FIRST METABOLIC PERIOD, BASAL DIET 2, TABLE 2					
N intake, gm/day	7.50	7.58	6.88	7.51	7.34
Urinary N, gm/day	3.56	4.97	3.98	5.06	4.51
Fecal N, gm/day	1.34	1.25	1.29	1.24	1.49
N excreted, gm/day	4.90	6.22	5.27	6.30	6.00
N retained, gm/day	+ 2.60	+ 1.36	+ 1.61	+ 1.21	+ 1.34
SECOND METABOLIC PERIOD, BASAL DIET 2, TABLE 2 PLUS 0.9 GM LYSINE [2]					
N intake, gm/day	7.97	8.46	7.88	7.94	7.75
Urinary N, gm/day	6.20	5.30	5.67	5.25	5.28
Fecal N, gm/day	1.34	1.59	1.23	1.54	1.53
N excreted, gm/day	7.54	6.89	6.90	6.79	6.81
N retained, gm/day	+ 0.43	+ 1.57	+ 0.98	+ 1.15	+ 0.94

[1] All values are average daily values determined by analysis of pooled daily aliquots for each period.

[2] Nitrogen intake includes 0.17 gm nitrogen derived from lysine.

during which lysine was given, the average retention was only 1.01 gm per day. All subjects except No. 2 showed an average daily nitrogen retention less than that of the first period. It is evident that the amount of protein provided by the unsupplemented "basal" diet was sufficient to cause a strong nitrogen balance. During the second period the subjects were approaching nitrogen equilibrium. No conclusion can be reached regarding the possible effect of lysine supplementation.

Experiment 2

The strong positive nitrogen balance obtained during the first metabolic period in experiment 1 indicated that the total protein intake was probably too high to demonstrate an effect of lysine supplementation. In experiment 2 the protein intake during all periods was reduced. The "depletion" diet (diet 3, table 2) providing 2.72 gm of nitrogen and about 2000 Cal. per person per day was given to all subjects for 4 days. Urinary nitrogen was determined on the 4th day. The "basal" diet (diet 4, table 2: 3.86 gm of nitrogen and about 2250 Cal. per person per day) was initiated on the 5th day and the first 5-day metabolic period began on the 6th day. The 8 subjects (table 1) participating in this experiment were divided according to body weights into two groups of 4 each. During the first metabolic period those in group A received a daily supplement of 0.9 gm of lysine, administered as described above, while the subjects in group B were maintained on the "basal" diet alone. During the second metabolic period group B received the lysine supplement, while group A received the "basal" diet alone.

Three of the 8 subjects lost small amounts of weight during the course of the experiment and 4 gained slightly suggesting that the caloric content of the diets was adequate for these subjects.

During the first metabolic period the 4 subjects receiving the lysine supplement had an average daily retention of 0.38 gm of nitrogen compared to an average loss of 0.07 gm in group B, a difference of 0.45 gm per day (table 4). Daily nitrogen balances were calculated for each individual using the daily urinary nitrogen values and the average values for nitrogen intake and fecal nitrogen. Of the total of 19 daily values for group A (one day's urine sample for subject 6 was lost), only three negative balances were obtained. Subjects of group B showed 11 days of negative balance.

When the lysine supplement was withdrawn during the second metabolic period, group A showed an average daily

TABLE 4

Nitrogen balance with and without lysine supplementation of an all-vegetable protein diet (experiment 2)[1]

	SUBJECTS							
	6	7	8	9	10	11	12	13
LAST DAY OF DEPLETION PERIOD, DIET 3, TABLE 2								
N intake, gm/day	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72
Urinary N, gm/day	3.31	1.66	2.62	2.41	3.25	1.87	2.70	3.20
FIRST METABOLIC PERIOD, BASAL DIET 4, TABLE 2								
	GROUP A, 0.9 GM LYSINE DAILY					GROUP B, NO SUPPLEMENT		
N intake, gm/day	4.10	4.10	4.10	4.10	3.93	3.93	3.93	3.93
Urinary N, gm/day	3.05[2]	2.77	2.76	2.91	2.81	2.84	2.88	3.05
Fecal N, gm/day	1.10	1.14	0.56	0.61	1.11	1.06	1.10	1.13
N excreted, gm/day	4.15	3.91	3.32	3.52	3.92	3.90	3.98	4.18
N retained, gm/day	— 0.05	+ 0.19	+ 0.78	+ 0.58	+ 0.01	+ 0.03	— 0.05	— 0.25
N average retained, gm/day		+ 0.35				— 0.06		
SECOND METABOLIC PERIOD, BASAL DIET 4, TABLE 2								
	GROUP A, LYSINE SUPPLEMENT WITHDRAWN					GROUP B, 0.9 GM LYSINE DAILY		
N intake, gm/day	3.79	3.79	3.79	3.79	3.96	3.96	3.96	3.96
Urinary N, gm/day	2.95	2.58	2.80	2.98	2.71	2.68	2.69	2.72
Fecal N, gm/day	1.22	1.42	1.05	0.60	1.02	1.04	1.09	1.24
N excreted, gm/day	4.17	4.00	3.85	3.58	3.73	3.72	3.78	3.96
N retained, gm/day	— 0.38	— 0.21	— 0.06	+ 0.21	+ 0.23	+ 0.24	+ 0.18	0.00
N average retained, gm/day		— 0.11				+ 0.16		

[1] Dietary and fecal nitrogen determined on pooled aliquots for each period. Urinary nitrogen determined daily and values for each period averaged.
[2] Average for 4 days; sample for second day lost.

loss of 0.11 gm of nitrogen, or an average decrease in retention between the two periods of 0.49 gm of nitrogen per person per day. Lysine supplementation in group B increased the nitrogen retention 0.23 gm per day from — 0.07 gm to + 0.16 gm. During this period there were 14 values of negative nitrogen balance for group A, as compared with 5 values of negative nitrogen balance for group B. Thus, all subjects in group A showed decreased nitrogen retention when lysine was withdrawn, and all subjects of group B showed an increased retention upon supplementation with lysine. Average group values for nitrogen retention were negative in the absence of lysine and positive in its presence.

Experiment 3

Experiment 3 was designed to test the effect of lysine supplements to the same diet used in experiment 2, but after more severe depletion. There were again 8 subjects (see table 1). The "depletion" period was extended to 7 days, protein intake was approximately 10 gm per day and caloric intake about 1000 Cal. per day (diet 5, table 2). All subjects lost weight in amounts ranging from 1.1 to 2.2 kg during the week. At the end of the week subjects were divided into two comparable groups on the basis of initial weight and weight loss. During a 5-day metabolic period group A received the basal diet (diet 6, table 2) and a daily supplement of 0.9 gm of lysine administered as described above, while group B received the same diet and a daily glycine supplement equivalent in nitrogen to the lysine. Examination of the daily urinary nitrogen values indicated that the responses during this period were similar to those obtained in experiment 2. Instead of reversing the groups with respect to lysine supplementation, the effect of methionine supplementation was tested during the following 5-day metabolic period. All subjects received lysine (0.9 gm daily) and, in addition, two subjects from each group were given a daily supplement of 1.0 gm of methionine. The remaining subjects were given

TABLE 5

Nitrogen balance during the three metabolic periods of experiment 3

	SUBJECTS							
	6	12	14	11	8	15	10	7
DEPLETION PERIOD, DIET 5, TABLE 2								
N intake, gm/day	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70
Urinary N, gm/day	4.93	3.63	4.82	3.82	3.73	3.81	4.31	3.42
Fecal N, gm/day	0.10	0.92	0.80	0.72	0.60	0.91	0.50	0.88
N excreted, gm/day	5.03	4.55	5.62	4.54	4.33	4.72	4.81	4.30
N retained, gm/day	−3.33	−2.85	−3.92	−2.84	−2.63	−3.02	−3.11	−2.60
FIRST 5-DAY METABOLIC PERIOD WITH BASAL DIET 6, TABLE 2								
	GROUP A, 0.9 GM LYSINE DAILY				GROUP B, GLYCINE SUPPLEMENT ONLY			
N intake, gm/day	4.13	4.13	4.13	4.13	4.13	4.13	4.13	4.13
Urinary N, gm/day	3.19	2.65	3.40	2.79	3.12	3.01	3.07	2.73
Fecal N, gm/day	0.65	1.07	0.84	1.03	1.33	1.42	1.12	1.23
N excreted, gm/day	3.84	3.72	4.24	3.82	4.45	4.43	4.19	3.96
N retained, gm/day	+0.29	+0.41	−0.11	+0.31	−0.32	−0.30	−0.06	+0.17
N average retained, gm/day	+0.22				−0.13			
SECOND 5-DAY METABOLIC PERIOD WITH BASAL DIET 6, TABLE 2 [1]								
	L + G	L + G	L + M	L + M	L + M	L + M	L + G	L + G
N intake, gm/day	4.21	4.21	4.21	4.21	4.21	4.21	4.21	4.21
Urinary N, gm/day	3.51	3.04	3.23	2.69	2.74	2.50	2.69	2.51
Fecal N, gm/day	0.61	1.08	0.87	0.97	1.16	1.13	1.21	1.35
N excreted, gm/day	4.12	4.12	4.10	3.66	3.90	3.63	3.90	3.86
N retained, gm/day	+0.09	+0.09	+0.11	+0.55	+0.31	+0.58	+0.31	+0.35

[1] Supplements during this period indicated by initials at top of each column; L = lysine, G = glycine, M = methionine.

glycine to furnish nitrogen equivalent to that provided by methionine. In table 5 the nitrogen balance for each subject during each of the three periods is summarized.

During the "depletion" period the average daily nitrogen loss was 3.04 gm, an average total loss of 21.28 gm of nitrogen per subject. Individual losses for the week ranged from 18.20 gm to 27.44 gm of nitrogen. The average urinary nitrogen values for the 7 consecutive days of depletion were: 6.33, 4.84, 4.31, 3.79, 3.42, 2.99, and 2.74 gm.

During the following 5 days in which the "basal" diet providing 3.96 gm of nitrogen daily was given, the 4 subjects receiving 0.9 gm of lysine (0.17 gm of nitrogen) daily had an average retention of 0.22 gm of nitrogen per day; the 4 subjects receiving only glycine lost on the average 0.13 gm of nitrogen per person per day. The mean difference in retention between the two groups was 0.35 gm. These results may be compared with the difference of 0.45 gm of nitrogen observed during the first metabolic period of experiment 2. The most negative balance observed in the absence of lysine was — 0.32 gm of nitrogen per day (subject No. 8).

The average nitrogen balance for group A during "depletion" was — 3.23 gm of nitrogen per day. The average retention of 0.22 gm of nitrogen with the lysine-supplemented basal diet represented an improvement in retention of 3.45 gm of nitrogen per day. Similar averages for group B were — 2.84 gm and — 0.13 gm of nitrogen during "depletion" and the first basal period, respectively. The improved retention shown by this group averaged only 2.71 gm of nitrogen per day. Daily individual balances showed 5 values of negative balances in group A compared to 14 in group B. The data confirm experiment 1. Although the subjects had been depleted to a greater extent than in experiment 2, the effect of lysine supplementation was no more marked.

The effect of methionine supplementation on nitrogen retention was evaluated by comparing the pairs of individuals whose previous diets had been the same. As shown in table 6, subjects 6 and 12, who received only lysine during both

periods, had an average decrease in retention of 0.26 gm of nitrogen per day during the second period (difference between averages for two periods). In subjects 11 and 14, who received lysine during both periods and methionine during the second, the average nitrogen retention increased by 0.23 gm per day per person. The difference between the averages for these two pairs of subjects (+ 0.49 gm of nitrogen per day) could be attributed to methionine. Comparing subjects 8 and 15 with subjects 7 and 10 in like manner, methionine alone

TABLE 6

Effect of methionine supplementation on nitrogen balance

Experiment 3

GROUP	SUBJECTS	AVERAGE NITROGEN RETENTION		AVERAGE CHANGE IN NITROGEN RETENTION
		Second period	Third period	
		(grams per day)[1]		*(grams per day)*
A	6 and 12	+ 0.35 (L)	+ 0.09 (L + G)	— 0.26
	11 and 14	+ 0.10 (L)	+ 0.33 (L + M)	+ 0.23
			Difference due to methionine	+ 0.49
B	8 and 15	— 0.31 (G)	+ 0.44 (L + M)	+ 0.75
	7 and 10	+ 0.05 (G)	+ 0.33 (G + L)	+ 0.28
			Difference due to methionine	+ 0.47

[1] Letters in parentheses indicate supplement: L = lysine, G = glycine, M = methionine.

accounted for an improved retention between the two periods of 0.48 gm of nitrogen per day. Thus, methionine in the presence of lysine appears to have caused an improvement in the utilization of the protein of the diet. Comparing the overall response of group A with group B, the combined effect of lysine and methionine appears to be greater than that of lysine alone.

Amino acid intakes

The results of the amino acid analysis of the composite "basal diet" in experiment 2, which was essentially the

same as the diet used in experiment 3, together with more extensive data upon the lysine, methionine, and cystine contents of the diets used in experiment 3, are shown in table 7. It is apparent that in diets as limiting as those used in these experiments the intake of lysine exceeds that of Rose's ('49) estimated requirement. Only in the depletion diet in experiment 3 was the value actually less. This diet supplied only 1000 Cal., contained no cereal and the protein sources were vegetables and fruits. The lysine content of

TABLE 7

Amino acid intakes compared to adult minimum requirements

AMINO ACID	EXP. 2	EXP. 3			ROSE'S ('49) MINIMUM DAILY REQUIREMENT
	Basal diet	Depletion diet	Basal diet 1st period	Basal diet 2nd period	
	(milligrams per person per day)				
Lysine	1227	761	1080	1040	800
Methionine	646	172	474	451	200 [1]
Cystine			426	473	900 [1]
Cystine and methionine			900	924	1100
Leucine	1117				1100
Valine	1072				800
Isoleucine	1398				700
Tryptophan	238				250
Threonine	1067				500
Phenylalanine	1624				1100

[1] These values calculated upon the basis of the statement (Rose, '53) that 80 to 85% of the methionine requirement previously estimated as 1100 mg/day is replaceable by cystine.

761 mg indicates that the mixed protein contained 7.2% of lysine, a value which compares favorably with many animal proteins.

The total cystine-methionine intake of approximately 900 mg per day is about 80% of the estimated minimum requirement. Recovery experiments made at the time the diets were analyzed gave average values of 112% for lysine, 80.8% for methionine, and 79% for cystine. Thus, it is probable that the actual methionine and cystine intakes were somewhat greater than indicated by the analytical data.

It is of some interest to compare the amino acid composition of the basal diet with that of high quality proteins. Table 8 shows a calculation on the "amino acid index" according to Block and Mitchell ('46–'47) in which the composition of egg protein is assumed to be ideal and the relative deficits of each of the amino acids in other proteins are calculated. This analysis indicates that methionine is the most limiting amino acid, but the deficiencies of lysine, tryptophan, total sulfur

TABLE 8

Comparisons of the essential amino acid composition of the all-vegetable basal diet, egg protein and amino acid requirements

AMINO ACID	PROTEIN OF EGG [1]	PROTEIN OF VEGETABLE DIET [2]	DIFFERENCE	DEFICIT	RATIO TO TRYPTOPHAN		
					Egg protein	Diet [2] protein	Rose's minimum
	%	%		%			
Lysine	7.2	4.50	— 2.70	— 38	4.8	4.5	3.2
Methionine	4.1	1.92	— 2.18	— 53	2.7	1.9	0.8
Cystine	2.4	1.87	— 0.53	— 22	1.6	1.9	3.6
Cystine and methionine	6.5	3.80	— 2.70	— 42	4.3	3.8	4.4
Leucine	9.2	4.65	— 4.55	— 49	6.1	4.7	4.4
Valine	7.3	4.47	— 2.83	— 39	4.9	4.5	3.2
Isoleucine	8.0	5.83	— 2.17	— 27	5.3	5.8	2.8
Tryptophan	1.5	0.99	— 0.51	— 34	1.0	1.0	1.0
Threonine	4.9	4.44	— 0.46	— 9	3.2	4.4	2.0
Phenylalanine	6.3	6.77	+ 0.47	+ 8	4.2	6.8	4.4

[1] Block and Mitchell ('46–'47).

[2] See table 7.

amino acids, leucine and valine are of the same order of magnitude. In the same table we have also compared the amounts of the amino acids relative to tryptophan in the basal diets, in egg protein and in Rose's minimum requirements. On this basis also the sulfur amino acids appear limiting. The analysis indicates that the over-all biological value of the dietary protein might be expected to be about 50% compared to egg protein, but that the ratios among the essential amino acids are not grossly abnormal. Whether amino acid imbalance (Elvehjem and Krehl, '51) i.e., excesses

of certain amino acids, becomes of any importance is not clear, but may be considered to be unlikely. If not, a biological value of 50% only indicates that twice as much protein must be fed to equal the requirements compared to egg protein. Since the absolute minimum need has been repeatedly estimated (Brody, '45) to be of the order of 1.4 to 2 mg of nitrogen per basal calorie, which should be met by an equivalent amount of high quality protein, the minimum protein requirement of these relatively small young women is estimated to be between 11 and 16 gm per day. ($1.42\ m^2 \times 24$ hours $\times$ 37 Cal./hour $\times$ 1.4 mg N $\times$ 6.25 = 11 gm of protein.) Thus, from 22 to 32 gm of protein from the basal diet may be assumed to meet minimum requirements. This type of calculation is at least consistent with the observations.

DISCUSSION

The performance of these subjects upon all-vegetable diets confirms previous experience (Hegsted et al., '46; Bricker et al., '45; Mitchell, '48). Nitrogen balance is readily achieved upon all-vegetable diets even without severe nitrogen depletion prior to the test. A considerable degree of dietary limitation is required to achieve the diet used in the first experiment which gave a protein intake of 48 gm per day. Upon this diet a strong positive balance was obtained during the first 5 days and equilibrium with lesser retentions was approached in the second 5 days. The basal diets used in the other experiments supplied only 25 gm of protein per day, yet the degree of negative balance obtained is not particularly striking. One of us has previously discussed the validity of balance studies as a measure of requirements (Hegsted, '52, '54) with the conclusion that the method is not a valid one. In spite of this, such data as are presented in this paper are not impressive as to the inadequacies of a vegetable diet in terms of protein. The fact remains that it is difficult to obtain a mixed vegetable diet which will produce an appreciable loss of body nitrogen without resorting to high levels of

sugar, jams, and jellies, and other essentially protein-free foods.

The diet used in the depletion period of experiment 3 contained only 10 gm of protein per day and was severely limited in calories. Weight loss was rapid in the subjects. Such diets are of no interest from the standpoint of protein or amino acid requirements, but it would be of interest to know whether the calories or nitrogen was the principal determining factor in the negative nitrogen balance produced.

Comparing our data with the figures Rose has presented on amino acid requirements there would appear to be no limiting amino acids in the diet. Cystine plus methionine may be slightly low but probably not outside the accuracy of the requirement figure. It should be recalled that Rose's figures are apparently based upon the individual tested which seemed to show the greatest need for the amino acid. The reasons for this procedure are obvious, but the statistical validity of it may be debated. In any event it must presumably mean that the estimates of Rose tend to be on the high side. Average requirements for threonine, valine, and tryptophan obtained by Leverton ('54) are lower than those of Rose.

In comparing the present data with the estimated requirements, one must consider the experimental approach. The Illinois workers gradually approached the minimum level by repeatedly lowering the intake of the amino acid under study. What happened to the nitrogen stores of these subjects during this period is not clear. If our own concept of nitrogen balance is correct, we assume that the requirement for an amino acid, as measured by balance, would gradually decrease as a subject's nitrogen stores were depleted. Whether this was an important variable in the studies is not clear to us, but presumably the procedure was reasonably standardized. In any event when nitrogen balance is obtained at intakes of amino acids totalling something like 20 gm of protein the subjects must be considered as "adapted" to a low level of protein intake.

Our own studies, although pertinent to the question of amino acid requirement, are more in the nature of "biological value" determinations on the dietary protein. The subjects were partially depleted of protein (this is obviously necessary for any studies upon either requirements or biological value) and then fed the test protein. It would be expected that proteins of high biological value would replace the previous nitrogen loss more rapidly than proteins of low biological value. This is the basis of the extensive work by Allison and co-workers ('46). Although our observations are not extensive they are consistent. We feel that they demonstrate an improvement in the biological value of the protein of the diet used by the addition of lysine and probably more so by the addition of lysine and methionine. The responses with lysine supplementation in no sense contradict Rose's estimated requirement and, the apparently greater response obtained with the further addition of methionine is entirely in agreement with the values upon estimated requirement.

The importance of the responses obtained may be debated. Rose's recommended intakes ('49) are twice the observed minimum. Considering the level of protein intake in these experiments, the degree of negative balance achieved, and the responses obtained with amino acid additions, we are not greatly impressed with the likelihood of protein or amino acid deficiencies in adult populations largely restricted to vegetable proteins. The recent publication of Widdowson and McCance ('54) where good growth, lack of evidence of nutritional deficiency, and no response to milk supplements was found in school children restricted to nearly all vegetable diets consisting largely of wheat flours, seems impressive in the same direction. In view of this evidence, the responses obtained by Albanese et al. ('55) on the addition of lysine to milk diets of infants is extremely interesting and deserves further study. Our own data are based upon young adults in good health. The extent to which the results may apply to the population in general is, of course, unknown.

The present studies emphasize an important and relatively unexplored area. Many are accustomed to speak and think of vegetable proteins as synonymous with cereal proteins. The possibility that the relatively minor amounts of protein supplied by fruits and vegetables might be important in supplementing the proteins of cereal diets has not been adequately considered. Of the constituents used in the vegetable diet, potato appears to have high quality protein. However, it cannot be emphasized too strongly that the total dietary protein must be considered as a unit in attempts to evaluate the protein or amino acid problems in nutrition.

SUMMARY

Nitrogen intake and excretion data for young adult subjects consuming all-vegetable diets with and without supplements of lysine and methionine are presented. The essential amino acids of the diet were determined.

An all-vegetable diet supplying 25 gm of protein per day of which 48% of the protein came from wheat products and 62% from cereals, contained all of the essential amino acids in quantities equaling or exceeding Rose's estimated minimum requirements. The amount of methionine plus cystine barely equaled the estimated requirement.

Supplementation of the diet with lysine improved the nitrogen retention following a depletion period and methionine plus lysine supplements appeared to improve the retention still more. These data are interpreted as demonstrating improvements in biological value of the dietary protein. Considering the relative ease with which the subjects approach nitrogen equilibrium on the unsupplemented diet, the likelihood of protein or amino acid deficiencies in an adult population consuming diets essentially of vegetable origin does not appear to be very great.

The data emphasize the probability that the proteins of fruits and vegetables which are usually not considered to be of importance in protein nutrition may be an important supplement to the cereal proteins.

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